Reactive Oxygen Species-induced Phosphorylation of p53 on Serine 20 Is Mediated in Part by Polo-like Kinase-3*

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Upon exposure of cells to hydrogen peroxide (H₂O₂) phosphorylation of p53 was rapidly induced in human fibroblast GM00637, and this phosphorylation occurred on serine 9, serine 15, serine 20, but not on serine 392. In addition, H₂O₂-induced phosphorylation of p53 was followed by induction of p21, suggesting functional activation of p53. Induction of phosphorylation of p53 on multiple serine residues by H₂O₂ was caffeine-sensitive and blocked in ATM−/− cells. Polo-like kinase-3 (Plk3) activity was also activated upon H₂O₂ treatment, and this activation was ATM-dependent. Recombinant His₆-Plk3 phosphorylated glutathione S-transferase (GST)-p53 fusion protein but not GST alone. When phosphorylated in vitro by His₆-Plk3, but not by the kinase-defective mutant His₆-Plk3K52R, GST-p53 was recognized by an antibody specifically to serine 20-phosphorylated p53, indicating that serine 20 is an in vitro target of Plk3. Also serine 20-phosphorylated p53 was coimmunoprecipitated with Plk3 in cells treated with H₂O₂. Furthermore, although H₂O₂ strongly induced serine 15 phosphorylation of p53, it failed to induce serine 20 phosphorylation in Plk3-deficient Daudi cells. Ectopic expression of a Plk3 dominant negative mutant, Plk3K52R, in GM00637 cells suppressed H₂O₂-induced serine 20 phosphorylation. Taken together, our studies strongly suggest that the oxidative stress-induced activation of p53 is at least in part mediated by Plk3.

Reactive oxygen species (ROS), ubiquitous present, are very reactive and cause damage to biological molecules, including DNA. ROS are potentially mutagenic and may be involved in activation of protooncogene and inactivation of tumor suppressor genes (1, 2). Thus, ROS are suspected to represent important human carcinogens (3, 4). Oxidative signals, either external or internal, are thought to be detected by sensor molecules and mediated by cellular signal transduction systems, which eventually result in cell cycle arrest, senescence, or apoptosis in normal diploid fibroblast cells. ATM has been shown to be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: ROS, reactive oxygen species; IR, ionizing radiation.
overrides the induction of G2 arrest by DNA damage (28).

We have been studying the biological role of polo-like kinase-3 (Plk3, previously named Prk) during normal and abnormal cell growth (17, 25, 30, 31). Here we report that ROS induces activation of Plk3 as well as p53, which is correlated with p53 phosphorylation on multiple serine sites. Activation of both Plk3 and p53 is ATM-dependent. In addition, we have obtained experimental evidence strongly suggesting that Plk3 mediates ROS-induced serine 20 phosphorylation of p53.

MATERIALS AND METHODS

Cell Culture—Various cell lines, including ATM-deficient cell line (ATCC number CRL-1702), were purchased from ATCC. CRL-1702 has been characterized as ATM-/- (32). GM00637 cell line (human fibroblast) was originally from the Coriell Institute for Medical Research. HeLa, A549, GM00637, DU145, LNCap, and PC-3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml penicillin and 50 μg/ml streptomycin sulfate) with 5% CO2. DAMI, HEL, and HL-60 cells were cultured in RPMI 1640 medium, and Daudi cells were culture in McCoy’s medium supplemented with fetal bovine serum and antibiotics as above.

Immunoblotting—Cells treated with H2O2 (200 μM unless otherwise specified) or adriamycin (100 μM) were collected and lysed (25). In some experiments, caffeine (2 μM) was supplemented to the cultured cells for 30 min prior to the treatment with H2O2 or adriamycin. Equal amounts (40 μg) of protein lysates from the treated cells were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies (New England Biolabs) to phosphorylated p53 (specifically phosphorylated on serine 9, serine 15, or serine 20) and p21, Bax, or α-tubulin. The same blots were also stripped and reprobed with antibodies to regular p53 (Santa Cruz Biotechnology). Signals were detected with horseradish peroxidase-conjugated goat secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Protein Kinase Assays—Immunocomplex kinase assays were performed essentially as described previously (25). In brief, A549 cells were exposed to H2O2 (200 μM) for various times, lysed, and subjected to immunoprecipitation with antibodies to Plk3. The resulting precipitates were resuspended in a kinase buffer (10 mM Hepes-NaOH (pH 7.4), 10 μM MnCl2, 5 mM MgCl2), and the kinase reaction was initiated by the addition of [γ32P]ATP (2 μCi) (Amersham Pharmacia Biotech) and casein (Sigma). After incubation for 30 min at 37°C, the reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Recombinant His6-Plk3, produced and purified as described previously (25, 30), was assayed for kinase activity as a positive control. In some kinase assays, GST-p53 was incubated with His6-Plk3 or His6-Plk3D301N in the kinase buffer supplemented with “cold” ATP. After reaction, Plk3-phosphorylated GST-p53 samples, as well as nonphosphorylated GST-Plk3, were blotted for serine 20 phosphorylation.

Coimmunoprecipitation Analysis—GM00637 cell lysates were incubated for 30 min at room temperature in a total volume of 500 μl with of 20 μl of protein A/G-agarose bead slurry (Santa Cruz Biotechnology). After removal of the beads, the supernatant was supplemented with either rabbit polyclonal (PharMingen) or mouse monoclonal antibodies to Plk3, or with control immunoglobulins followed by incubation with constant agitation for overnight at 4°C. Protein A/G-agarose beads (20 μl) were then added to each immunoprecipitation mixture, and the incubation was continued for 1 h at room temperature. Immunoprecipitates were collected by centrifugation, washed three times with the cell lysis buffer, and subjected to immunoblot analysis with monoclonal antibodies to serine 20-phosphorylated p53.

Transient Transfection—GM00637 cells were transfected, using the LipofectAMINE method (Life Technologies, Inc.), with constructs expressing Plk3 or Plk3D301N or with the vector pCR352. One day after transfection, cells were treated or without H2O2 for 30 min. Cell lysates were prepared and blotted for Plk3, p53, or serine 20-phosphorylated p53.

RESULTS

Although recent studies have shown that phosphorylation of p53 plays an important role in stabilization and activation of this tumor suppressor protein in cells exposed to ionizing radiation (IR) or UV (6, 33), the mechanism by which ROS-induced p53 accumulation/activation remains unclear (34). To determine whether ROS activates p53 through phosphorylation, GM00637 cells were exposed to H2O2 for various times, and p53 phosphorylation status was analyzed by immunoblotting using phospho-specific antibodies. Fig. 1A shows that upon H2O2 treatment, p53 was rapidly phosphorylated on serine 20 and serine 15 in GM00637D cells. Serine 9 phosphorylation was also induced with a slow kinetics (Fig. 1A, lane 5). However, serine 392 phosphorylation was not detected (data not shown). These results indicate that p53 accumulation upon oxidative stress as reported by von Harsdorf and Dietz (35) is at least partly due to phosphorylation of p53 on serine 15 and serine 20, because these two residues are located within the domain of the protein that interacts with HDM2 (human ortholog of murine double minute-2 protein, MDM2), resulting in stabilization of the normally short-lived p53 protein in response to the stress (36).

Phosphorylation and activation of p53 upon challenge with genotoxic stress such as IR and UV often results in cell cycle arrest (6). In fact, the trans-activation by p53 of genes such as those encoding p21 and Bax proteins is thought to be responsible at least in part for cell cycle arrest and apoptosis, respectively, in cells subjected to genotoxic stress (37). To determine whether H2O2-induced p53 phosphorylation is correlated with its functional activation, we measured expression of its target genes p21 and Bax. Fig. 1B shows that 5 h after H2O2 treatment, the p21 protein level began to increase (lane 2) and that 7 h post-treatment it was more than quadrupled (lane 3) compared with the untreated control (lane 1). On the other hand, little increase in Bax protein levels were observed in cells treated with H2O2.

DNA damage caused by IR activates p53 through phosphorylation on multiple residues and this activation is ATM-dependent (33). To determine whether ROS-induced p53 phosphorylation was also ATM-dependent, we treated GM00637 cells with caffeine, an ATM/ATR inhibitor, prior to exposure of the cells to H2O2, or IR-mimetic drug adriamycin. Fig. 2A shows that caffeine (CFN) partially blocked H2O2-stimulated phosphorylation of p53 on serine 15 and serine 20 (lanes 2 and 5), whereas it completely inhibited adriamycin (ADR)-induced phosphorylation of p53 on all three residues (lanes 3 and 6).
These observations suggest that p53 activation by ROS is at least in part dependent on ATM and/or ATR. To further confirm that ATM was important in mediating p53 phosphorylation by H₂O₂, we exposed ATM-deficient CRL-1702 cells to H₂O₂. Fig. 2B shows that in the ATM-deficient cells after H₂O₂ treatment enhancement in phosphorylation of p53 on neither serine 20 nor serine 15 was observed, indicating that ROS-induced phosphorylation and activation of p53 is ATM-dependent.

Our laboratory has been studying human Plk3, which is involved in regulating cell cycle progression (17, 25, 30). As an initial step to identify protein kinase(s) responsible for phosphorylation of p53 induced by ROS, we examined the possibility that Plk3 phosphorylates the same residue of Cdc25C (serine 216) as that targeted by Chk1 and Chk2. Chk1 and Chk2 are also reported to phosphorylate p53 on serine 20 (38, 39). A549 cells, expressing good levels of Plk3, were treated with ROS for various times. Plk3 immunoprecipitated from the treated cells was analyzed for its kinase activity using casein as substrate as described previously (25). Fig. 3A shows that compared with the control (lane 1) Plk3 kinase activity was rapidly activated in A549 cells (lane 2) and maintained for at least 1 h. To determine whether Plk3 activation was ATM-dependent, CRL-1702 cells treated with H₂O₂ were collected, and Plk3 immunoprecipitates were assayed for Plk3 kinase activity. Fig. 3B shows that whereas recombinant Plk3 phosphorylated casein effectively (lane 4), no difference in Plk3 kinase activity was detected between untreated control (lane 1) and H₂O₂-treated CRL-1702 cells (lane 2), suggesting that Plk3 activation also requires ATM. In addition, Plk3 activation was caffeine-sensitive because pretreatment of A549 cells with caffeine completely blocked activation of Plk3 by H₂O₂ (data not shown).

To determine the possibility that Plk3 was involved in mediating H₂O₂-induced p53 phosphorylation, we screened a dozen cell lines for Plk3 expression. We observed (Fig. 4A) that Daudi (B lymphoblastic leukemic cells with wild-type p53 (40)) did not express detectable levels of Plk3, whereas other tested cell lines expressed various levels of this protein. Further analysis with polymerase chain reaction confirmed that no Plk3 expression was detectable in Daudi cells (data not shown). To determine whether the absence of Plk3 expression affected p53 phosphorylation, we analyzed p53 phosphorylation on both serine 20 and serine 15 residues in Daudi cells exposed to H₂O₂. Fig. 4B shows that p53 phosphorylation on serine 15 is rapidly induced and maintained for at least 2 h in Daudi cells (lanes 2–5). In contrast, no serine 20 phosphorylation was observed. Chk2 is reported to phosphorylate p53 on serine 20 (39). Re-probing the same blot with antibody to Chk2 revealed that...
Daudi cells expressed abundant Chk2 (Fig. 4B). These observations suggest that Plk3 is involved in regulating serine 20 phosphorylation of p53.

We next asked whether Plk3 directly phosphorylated p53. In vitro kinase assays showed (Fig. 5A) that recombinant histidine-tagged Plk3 (His<sub>6</sub>-Plk3) phosphorylated GST-p53 (lane 2), as well as casein (lane 1), but not GST alone (lane 5), indicating that Plk3 targets the p53 moiety of GST-p53. A kinase-defective mutant of Plk3, His<sub>6</sub>-Plk3<sup>K52R</sup>, in which lysine 52 was replaced with arginine, did not significantly phosphorylate GST-p53 (Fig. 5A, lane 3). To further examine whether the serine 20 residue of p53 was a phosphorylation target of Plk3, we incubated GST-p53 with His<sub>6</sub>-Plk3 or His<sub>6</sub>-Plk3<sup>K52R</sup> in the kinase buffer supplemented with [γ-<sup>32</sup>P]ATP. GST and a casein were used as negative and positive controls, respectively. After kinase reaction, samples were fractionated on SDS-polyacrylamide gel electrophoresis followed by autoradiography. B, GST-p53 was phosphorylated in vitro by His<sub>6</sub>-Plk3 and His<sub>6</sub>-Plk3<sup>K52R</sup> in the kinase buffer supplemented with "cold" ATP. The reaction samples and protein lysates from H<sub>2</sub>O<sub>2</sub>-treated GM00637 cells (lane 1) were then blotted with the antibody to phosphoserine 20 of p53. Partial degradation of GST-p53 was observed (lane 5). C, equal amounts of protein lysates from GM00637 cells treated with or without H<sub>2</sub>O<sub>2</sub> were immunoprecipitated with the antibody to Plk3 or control IgGs. Immunoprecipitates were then blotted for serine 20-phosphorylated p53. GM00637 cell lysates were used as a positive control.

To further demonstrate that Plk3 regulated serine 20 phosphorylation of p53 in vivo, GM00637 cells were transfected with constructs expressing either Plk3 or Plk3<sup>K52R</sup>. One day after transfection, both Plk3 proteins were expressed (Fig. 6A, presence of serine 20-phosphorylated p53). Fig. 5C shows that neither the control IgGs appreciably precipitated serine 20-phosphorylated p53 from the H<sub>2</sub>O<sub>2</sub>-treated cells (lane 1) nor Plk3 antibody brought down the phospho-p53 from the untreated control cells (lane 2) However, Plk3 antibody precipitated p53 that was phosphorylated on serine 20 from cells treated with H<sub>2</sub>O<sub>2</sub>.

To explore the physical interaction between p53 and Plk3, we immunoprecipitated Plk3 from cells treated with or without H<sub>2</sub>O<sub>2</sub>, and Plk3 immunoprecipitates were then blotted for the
amount of toxic ROS in some cells (33). Thus, a feedback loop between p53 and ROS may exist, which is presumably to amplify the stress signal, resulting in accelerated programmed cell death when damage caused by a genotoxic stress is beyond repair.

Recent advances indicate that reversible phosphorylation plays an important role in the DNA damage checkpoint activation. In fact, p53 is rapidly phosphorylated upon exposure of cells to IR or UV (6). Our current studies demonstrated that oxidative stress activates p53 also through phosphorylation on multiple residues. The kinetics of ROS-induced phosphorylation of p53 on various serine residues is apparently different (Fig. 1A), suggesting the involvement of several protein kinases. It is also likely that phosphorylation of certain residues may facilitate the subsequent phosphorylation of other residues. Consistent with the latter scenario, phosphorylation of threonine 18 by casein kinase II requires prior phosphorylation of serine 15 by ATM upon DNA damage (41).

Our current studies indicate that Plk3 is directly involved in $H_2O_2$-induced phosphorylation of p53 on the serine 20 residue. First, induction of both p53 phosphorylation and Plk3 kinase activity by $H_2O_2$ is ATM-dependent (Figs. 2B and 3B). Second, $H_2O_2$ does not induce serine 20 phosphorylation of p53 in Daudi cells that express Chk2 but no detectable levels of Plk3 (Fig. 4). Third, Plk3, but not Plk3K52R, directly phosphorylates GST-p53 (but not GST alone) in vitro, and Plk3-phosphorylated GST-p53 contains a strong serine 20 epitope (Fig. 5). Fourth, Plk3 interacts with serine 20-phosphorylated p53 when cells are exposed to $H_2O_2$ (Fig. 5C). Fifth, ectopic expression of Plk3, but not the kinase-defective mutant Plk3K52R, results in significantly enhanced phosphorylation of p53 on serine 20 after $H_2O_2$ treatment.

Our studies, together with previous observations (30), suggest that Plk3 may act in parallel with Chk1 and Chk2, downstream of ATM or ATR. Plk3 may preferentially transduce signals generated by a specific genotoxic stress such as $H_2O_2$, just as Chk1 and Chk2 are differentially activated by UV radiation and IR, respectively (6). The observation that serine 20 phosphorylation of p53 was not induced by $H_2O_2$ in Daudi cells that express abundant Chk2 but no detectable Plk3 supports this notion. On the other hand, given that Cdc5 acts downstream of Rad53 in yeast (26), it is also possible that Plk3 may lie downstream of Chk2 (and/or Chk1). Plk3 may integrate the signals from ATM-Chk2 and ATR-Chk1 and induce cell cycle arrest or apoptosis by phosphorylating either Cdc25C on serine 216 or p53 on serine 20. Consistent with the latter scenario, Plk3 is activated by IR-mimetic drug adriamycin and UV radiation (data not shown) in addition to $H_2O_2$.

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DISCUSSION

The mechanism by which mammalian cells transmit signals in response to oxidative damage remains unclear. Here we report that ROS phosphorylates and activates p53 tumor suppressor protein. Consequences of p53 activation are either cell cycle arrest or apoptosis. We have observed that p53 activation in response to $H_2O_2$ treatment results in significant increase in expression of p21, but not of Bax (Fig. 1B), which is consistent with our observation that the concentration of $H_2O_2$ used in our experiments did not cause significant apoptosis of GM00637 cells (data not shown). However, we cannot exclude the possibility of Bax activation by post-translational mechanisms. Interestingly, it has been proposed that p53 may cause cell death by directly stimulating mitochondria to produce an excess

FIG. 6. Plk3 regulates serine 20 phosphorylation of p53 in vivo.
A, GM00637 cells were transfected with constructs expressing Plk3 or Plk3K52R or with the vector alone. One day after transfection, cells were lysed, and equal amounts of proteins from the transfected cells were blotted with the antibody to Plk3. Plk3 cells transfected with various constructs as indicated were treated with or without $H_2O_2$ (20 $\mu$M) for 30 min. Equal amounts of proteins from various treatments were blotted with antibodies to serine 20-phosphorylated p53 or $\alpha$-tubulin.

B, lanes 3 and 4) at a level higher than the endogenous one (the band with a slower mobility). The fast mobility of both transfected Plk3 proteins was due to a short truncation at the amino terminus. Further analysis of the transfected cells showed (Fig. 6B) that no significant enhancement in serine 20 phosphorylation was detected when cells were transfected with either Plk3 (lane 2) or Plk3K52R (lane 3) compared with cells transfected with vector alone (lane 1). However, when Plk3-transfected cells were exposed to a low concentration of $H_2O_2$, a significant increase in serine 20 phosphorylation was detected (lanes 2 and 5). In contrast, no such enhancement in serine 20 phosphorylation was detected in cells transfected with Plk3K52R (lanes 3 and 6). These observations suggest that Plk3 needs to be activated by ROS before it can fully phosphorylate its physiological substrates.
Activation of p53 by ROS

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