ATM Mediates Phosphorylation at Multiple p53 Sites, Including Ser46, in Response to Ionizing Radiation*

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The p53 tumor suppressor protein preserves genome integrity by regulating growth arrest and apoptosis in response to DNA damage. In response to ionizing radiation (IR), ATM, the gene product mutated in ataxia telangiectasia, stabilizes and activates p53 through phosphorylation of Ser15 and (indirectly) Ser46. Here we show that phosphorylation of p30 on Ser46 is important for p3 apoptotic activity, as well as on Ser8, in response to IR also is dependent on the ATM protein kinase. IR-induced phosphorylation at Ser46 was inhibited by wortmannin, a phosphatidylinositol 3-kinase inhibitor, but not PD169316, a p38 MAPK inhibitor. p53 C-terminal acetylation at Lys320 and Lys382, which may inhibit, but not PD169316, a p38 MAPK inhibitor. p53R2, in response to IR also is dependent on the ATM protein kinase. IR-induced phosphorylation at Ser46 was inhibited by wortmannin, a phosphatidylinositol 3-kinase inhibitor, but not PD169316, a p38 MAPK inhibitor. p53 C-terminal acetylation at Lys320 and Lys382, which may stabilize p53 and activate sequence-specific DNA binding, required Ser15 phosphorylation by ATM and was enhanced by phosphorylation at nearby residues including Ser8, Ser9, and Thr18. These observations, together with the proposed role of Ser46 phosphorylation in mediating apoptosis, suggest that ATM is involved in the initiation of p53-dependent apoptosis after IR in human lymphoblastoid cells.

In response to DNA damage, the p53 tumor suppressor protein is phosphorylated on each of the seven serines and one threonine in the first 50 amino acids of its N-terminal transactivation domain as well as at several sites in its carboxyl (C)-terminal tetramerization/regulatory domain (1, 2). As a transcription factor, p53 induces or represses several genes

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that regulate cell cycle arrest, DNA repair or apoptosis, including p21WAF1, MDM2, GADD45, p53R2, and p53AIP1. Recent studies suggest that specific p53 phosphorylation events are important for the activation or repression of specific promoters (3–6). Optimal induction and activation of p53 after exposure to IR requires phosphorylation by the ATM protein kinase (1, 2, 7). ATM is thought to directly phosphorylate Ser15 in vivo (8, 9) and also is required for phosphorylation of Ser20 through activation of the Chk2 protein kinase, which phosphorylates Ser20 in vitro (10–12). However, the potential role for ATM in regulating p53 modifications at other sites has not previously been explored.

EXPERIMENTAL PROCEDURES

Cell Cultures and Inhibitors—Epstein-Barr virus immortalized normal (GM02254) and A-T (GM01526) human lymphoblast cultures were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). H1299 (ATCC CRL-5803), a human lung carcinoma cell line that is null for both TP53 alleles, and A549 (ATCC CCL-185), a human lung carcinoma cell line that expresses wild-type p53, were obtained from the American Type Culture Collection (Manassas, VA). All cells were grown in Dulbecco’s modified essential medium (Invitrogen) supplemented with 15% (lymphoblasts) or 10% (H1299) fetal bovine serum, 100 ng/ml penicillin/streptomycin in a humidified atmosphere with 5% CO2. Wortmannin (Sigma) was prepared as a 10 mM stock and PD169316 (Calbiochem, Inc.) as a 1 mM stock in Me2SO; both were stored at −20 °C and diluted into the cell media immediately before use.

Induction of DNA Damage, Immunoprecipitation, and Western Immunoblot Analyses—Asynchronously growing cultures of H1299 cells were irradiated using a Shepherd Mark I137Cs irradiator at a dose rate of 3.2 Gy/min. To detect p53 acetylation, the deacetylase inhibitor trichostatin A (Wako, Osaka, Japan) was added at a final concentration of 5 μM 4 h before harvesting. Cultures were harvested at the indicated times after treatment, washed twice with ice-cold phosphate-buffered saline, and lysed in ice-cold lysis buffer (50 mM Tris-HCl at pH 7.5, 5 mM pyrophosphate, 25 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride). Immunoprecipitation and Western blot analyses were performed as described (13, 14). Anti-p53 monoclonal antibody DO-1 was purchased from Santa Cruz Biotechnology, Inc. in all Western blot analyses, unless otherwise stated. Immunoprecipitation was confirmed by Coomasie Brilliant Blue staining of the SDS-polyacrylamide gels after transfer to the polyvinylidene difluoride membranes.

Phosphorylation- and Acetylation-specific p53 Antibodies—Rabbit polyclonal antibodies specific for p53 phosphorylated at Ser15, Ser46, Ser20, Ser37, and Thr18 or acetylated at Lys320 or Lys382 have been described (13–15). A similar approach was used to generate antibodies specific for p53 phosphorylated at Ser46, Ser15, or Ser292. Briefly, rabbit antibodies that recognize p53 phosphorylated at Ser46 (PAbSer(p)46), Ser15 (PAbSer(p)15), or Ser292 (PAbSer(p)292) were elicited against the human p53 sequences Ac41–51/46PC, Ac310–321/315PC or Ac-C-385–399PC coupled to keyhole limpet hemocyanin, respectively. Phosphorylation-specific antibodies were affinity-selected. The specificity of each antibody was confirmed by enzyme-linked immunosorbent assay using synthetically prepared p53 peptides and with immunoblot assays by probing GST-human p53 expressed in Escherichia coli.

Plasmids and cDNA Constructs—Wild-type and the L22Q/W238S mu
Identification of ATM-dependent p53 Phosphorylation—To determine whether other p53 posttranslational modifications depend on ATM, we prepared a panel of polyclonal antibodies that, respectively, recognize p53 modified at each of 12 sites at which it is phosphorylated or acetylated (13–15, 19). These antibodies were then used to examine the time course of phosphorylation at each site after exposure of normal (GM02254) and A-T (GM01526) human lymphoblasts to 8 Gy IR (Fig. 1). Acetylation at two C-terminal sites, Lys320 and Lys382, also was examined. p53 phosphorylation was observed at Thr18 in either normal or A-T lymphoblasts, consistent with a previous report (19). Phosphorylation at Thr18 may be cell line-dependent, and no IR-induced increase in phosphorylation was observed at Thr18 in either normal or A-T lymphoblasts. Likewise, phosphorylation at Ser399 is thought to occur primarily through activation of ATR after exposure to e.g. UV light (20), and phosphorylation of Ser37 was not observed in either the normal or A-T lymphoblasts. A very similar pattern of IR-induced p53 phosphorylation was observed in A549 lung and MCF7 breast carcinoma cell lines (data not shown), indicating that these modifications are not specific to lymphoblasts. In normal lymphoblasts, increased phosphorylation at Ser15, Ser20, and Ser46 (and Ser9, data not shown) is delayed in A-T cells compared with normal cells. The amount of p53 in A-T cells was less than half of that in normal cells up to 0.5 h after exposure and then increased to ~80% of that in normal lymphoblasts by 4 h after irradiation. Although low levels of constitutive phosphorylation were observed at Ser9, Ser15, Ser20, Ser37, and Ser46, phosphorylation increased rapidly at each of these sites and at Ser15, Ser19, Ser20, and Ser46, after exposure of normal lymphoblasts to IR. Phosphorylation at Thr18 may be cell line-dependent, and no IR-induced increase in phosphorylation was observed at Thr18 in either normal or A-T lymphoblasts. Likewise, phosphorylation at Ser399 is thought to occur primarily through activation of ATR after exposure to e.g. UV light (20), and phosphorylation of Ser37 was not observed in either the normal or A-T lymphoblasts. A very similar pattern of IR-induced p53 phosphorylation was observed in A549 lung and MCF7 breast carcinoma cell lines (data not shown), indicating that these modifications are not specific to lymphoblasts. In normal lymphoblasts, increased phosphorylation at Ser15, Ser20, and Ser46 (and Ser9, data not shown) is delayed in A-T cells compared with normal cells. The amount of p53 in A-T cells was less than half of that in normal cells up to 0.5 h after exposure and then increased to ~80% of that in normal cells at 4 h.

Unexpectedly, phosphorylation at Ser46 also was defective in A-T lymphoblasts after exposure to IR, and phosphorylation at Ser9 was reduced and delayed (Fig. 1, A and B). These data suggest that Ser9 and Ser46 also may be phosphorylated by an ATM-activated protein kinase (or possibly ATM itself); alternatively, in response to IR, these phosphorylations may depend upon prior phosphorylation of Ser15 or Ser20. In response to UV irradiation, Ser46 can be phosphorylated by the p38 MAPK (22), and recently Ser46 was reported to be phosphorylated by a homeodomain-interacting protein kinase 2 (HIPK2), which also is activated by exposure of cells to UV light (23, 24). The kinase that phosphorylates Ser46 after IR has not been identified. To further establish the importance of ATM in mediating phosphorylation of Ser46, we examined the effect of wortmannin, an inhibitor of ATM (25), and PD169316, a p38 MAPK-specific inhibitor (26), on Ser46 phosphorylation in A549 cells in response to IR. Fig. 2 shows that increasing concentrations of wortmannin inhibited IR-induced phosphorylation at both Ser15 and Ser46 consistent with dependence on ATM, while increasing concentrations of PD169316 had no effect on phosphorylation at either site. Similar results were obtained in BT normal lymphoblasts (data not shown). Phosphorylation at each of the sites shown in Fig. 1 after exposure to IR also was examined in two other pairs of normal and A-T lymphoblasts, C3ABR and AT24RM, and BT (normal) and L3 (A-T), with
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Similar results; in neither A-T cell line did IR induce phosphorylation of Ser15, while robust phosphorylation at this site was observed in normal lymphoblasts (data not shown). That the p53 in A-T cells is capable of being phosphorylated in response to DNA damage was shown by examining the response in GM02254 and GM01526 cells exposed to UV light. In this case, no significant difference in p53 phosphorylation between the normal and A-T lymphoblasts was observed (data not shown), indicating that phosphorylation at Ser9, Ser15, Ser20, and Ser46 was unlikely to be masked in A-T cells. These findings fit previous observations that ATM acts specifically in the cellular response to IR (7). Thus, the N-terminal p53 phosphorylation sites can be grouped into two categories with respect to dependence on ATM for rapid phosphorylation in response to IR. One group, consisting of Ser6, Ser33, Ser37, and Ser392, is independent of ATM and constitutively phosphorylated at low levels; the other group, consisting of Ser9, Ser15, Ser20, and Ser46, is ATM-dependent for a rapid response to IR-induced damage, presumably DNA double strand breaks.

Dependence of C-terminal Acetylation on N-terminal Phosphorylation—Previously, we and others suggested that acetylation of human p53 after DNA damage may be mediated through phosphorylation at N-terminal sites (4, 15, 27). To address this issue in vivo, we first examined the time course of p53 acetylation at Lys320 and Lys382 after exposure to 8 Gy IR in normal and A-T lymphoblasts (Fig. 3A). In normal lymphoblasts, acetylation at Lys320 was observed within 1 h after IR and at Lys382 by 2 h; both sites were well acetylated at 4 h after IR. In contrast, in A-T lymphoblasts, acetylation at both sites was significantly delayed and reduced (by 20–40%) at 4 h after IR compared with normal lymphoblasts (Fig. 3B). No differences in the acetylation of these sites was observed between normal and A-T lymphoblasts after UV irradiation (data not shown).

To determine which N-terminal phosphorylation site(s) are important for acetylation, we examined mutant p53s in which individual N-terminal phosphorylation sites were changed to alanine, and 30 min later cultures were irradiated with 10 Gy. Two hour postirradiation cultures were harvested for Western immunoblot analysis as described in the legend to Fig. 1. A, wortmannin was added at 10 (lane 3), 50 (lane 4), or 100 (lane 5) μM. B, PD169316 was added at 2.5 (lane 3), 5 (lane 4), or 10 (lane 5) μM.

FIG. 2. Phosphorylation at Ser15 and Ser46 is inhibited by wortmannin. Wortmannin (WM), an inhibitor of phosphatidylinositol-3-like kinases including ATM, or PD169316 (PD), a p38 MAPK-specific inhibitor, were added to A549 cultures as indicated, and 30 min later cultures were irradiated with 10 Gy. Two hour postirradiation cultures were harvested for Western immunoblot analysis as described in the legend to Fig. 1. A, wortmannin was added at 10 (lane 3), 50 (lane 4), or 100 (lane 5) μM. B, PD169316 was added at 2.5 (lane 3), 5 (lane 4), or 10 (lane 5) μM.

Staining with the polyclonal, p53-specific antibody Ab-7, and neuronal cells are much more sensitive to p53-dependent, IR-induced apoptosis, and both p53 and ATM are required for its induction (28, 29). We suggest that in lymphoid cells, activation of ATM in response to DNA double strand breaks mediates activation of an unidentified protein kinase that phosphorylates p53 at Ser46, possibly through recruitment of p53DINP1, the product of a recently identified p53-inducible gene. Previous studies showed that phosphorylation of p53 at Ser15 and Ser20 in response to IR is mediated by the ATM protein kinase and that these modifications are important for stabilizing and activating p53 as a transcription factor. We show here, for the first time, that phosphorylation of Ser9 and Ser46 also are dependent on the ATM kinase (Figs. 1 and 4). Phosphorylation of Ser46 was shown to be important for the induction of apoptosis in response to damage caused by exposure of epithelial-derived cell lines to UV light (6, 22), and two protein kinases capable of phosphorylating Ser46, p38 MAPK (22) and HIPK2 (23, 24), both of which are activated after exposure of cells to UV light, have been described. In contrast to UV light, activation of p53 after exposure of epithelial cells to IR primarily induces cell cycle arrest in G1. However, that in lymphoid and neuronal cells are much more sensitive to p53-dependent, IR-induced apoptosis, and both p53 and ATM are required for its induction (28, 29). We suggest that in lymphoid cells, activation of ATM in response to DNA double strand breaks mediates activation of an unidentified protein kinase that phosphorylates p53 at Ser46, possibly through recruitment of p53DINP1, the product of a recently identified p53-inducible gene.
gene that facilitates p53 phosphorylation at Ser46 in response to IR (30). This protein kinase is unlikely to be p38 MAPK, which phosphorylates Ser46 in response to UV, since PD169316, a p38 kinase-specific inhibitor, failed to block phosphorylation of Ser46 by UV (4).

We also found that ATM is required for phosphorylation of p53 at Ser3. We previously reported that Ser3 and Ser9 became strongly phosphorylated in response to both IR- and UV-induced DNA damage and suggested that Ser3 may be phosphorylated by CK1 in response to phosphorylation of Ser9 (13). In vitro CK1 phosphorylates serines and threonines two residues distal to a phosphorylated serine or threonine. The data presented here suggest that in response to IR, phosphorylation of Ser3 may be independent of phosphorylation at Ser9 and dependent upon activation of an unknown protein kinase by ATM. Thus, as for Ser9, the kinase that phosphorylates Ser3 in response to IR is likely to be activated by ATM. We cannot, however, rule out the possibility that recognition of Ser3 by its kinase requires either Ser15 or its phosphorylation.

The functional consequences of phosphorylation at Ser9 and Ser3 are unknown. Changing either serine to alanine had little effect on the ability of chimeric Gal4-p53(1–27) showing that phosphorylation of Ser15 recruits CBP/p300 to transcription of a reporter in transient transfection assays (4).

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