Ionizing Radiation Activates Nuclear Protein Phosphatase-1 by ATM-dependent Dephosphorylation*

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Ionizing radiation (IR) is known to activate multiple signaling pathways, resulting in diverse stress responses including apoptosis, cell cycle arrest, and gene induction. IR-activated cell cycle checkpoints are regulated by Ser/Thr phosphorylation, so we tested to see if protein phosphatases were targets of an IR-activated damage-sensing pathway. Jurkat cells were subjected to IR or sham radiation followed by brief \(^{32}P\) metabolic labeling. Nuclear extracts were subjected to microcystin affinity chromatography to recover phosphatases, and the proteins were analyzed by two-dimensional gel electrophoresis. Protein sequencing revealed that the microcystin-bound proteins with the greatest reduction in \(^{32}P\) intensity following IR were the \(\alpha\) and \(\delta\) isoforms of protein phosphatase 1 (PP1). Both of these PP1 isoforms contain an Arg-Pro-Ile/Val-Thr-Pro-Pro-Arg sequence near the C terminus, a known site of phosphorylation by Cdc/Cdk kinases, and phosphorylation attenuates phosphatase activity. In wild-type Jurkat cells or ataxia telangiectasia (AT) cells that are stably transfected with full-length ATM kinase, IR resulted in net dephosphorylation of this site in PP1 and produced activation of PP1. However, in AT cells that are deficient in ATM, IR failed to induce dephosphorylation or activation of PP1. IR-induced PP1 activation in the nucleus may be a critical component in an ATM-mediated pathway controlling checkpoint activation.

Materials and Methods

Cell Culture—Jurkat cells (a human T cell lymphoma cell line) were grown in RPMI 1640 medium (Life Technologies, Inc.) with penicillin and streptomycin and 10% fetal bovine serum. FT/pEBST and FT/pEBST-YZ5 cell lines were both derived from the AT22JE-T line (17), an immortalized fibroblast line containing a homozygous frameshift mutation at codon 762 of the ATM gene. AT22JE-T cells were transfected with the mammalian expression vector pEBST (18) containing either the hygromycin resistance marker to yield FpEBST cells or with full-length ATM open reading frame to yield FpEBST-YZ5 cells. FT/pEBST and FT/pEBST-YZ5 were generously provided by Y. Shiloh (Tel Aviv University) and grown in Dulbecco’s modified Eagle’s medium with 15% fetal bovine serum and 10 \(\mu\)g/ml hygromycin B. All cells were grown in RPMI 1640 medium (Life Technologies, Inc.) with penicillin and streptomycin and 10% fetal bovine serum. FT/pEBS7 and FT/pEBS7-YZ5 cells were both derived from the AT22JE-T line (17), an immortalized fibroblast line containing a homozygous frameshift mutation at codon 762 of the ATM gene. AT22JE-T cells were transfected with the mammalian expression vector pEBST (18) containing either the hygromycin resistance marker to yield FpEBST cells or with full-length ATM open reading frame to yield FpEBST-YZ5 cells. FT/pEBST and FT/pEBST-YZ5 were generously provided by Y. Shiloh (Tel Aviv University) and grown in Dulbecco’s modified Eagle’s medium with 15% fetal bovine serum and 10 \(\mu\)g/ml hygromycin B. All cells were grown in RPMI 1640 medium (Life Technologies, Inc.) with penicillin and streptomycin and 10% fetal bovine serum.

Preparation of Nuclear Extracts—Cells were collected by centrifugation in an ice-cold preparation buffer consisting of 20 \(\mu\)M HEPES pH 7.4, 110 \(\mu\)M potassium acetate, 2 \(\mu\)M magnesium acetate, and 5 \(\mu\)M EGTA. The cell pellet was resuspended in the same buffer containing 1 \(\mu\)g/ml aprotinin, 1 \(\mu\)M PefablocSC, 0.2 \(\mu\)M PMSF, and 1 \(\mu\)M dithiothreitol at 5 \(\times\)10^6 cells/ml. Digitonin was added to a final concentration of 50 \(\mu\)g/ml to permeabilize the plasma membrane and release the cytosol. The cell suspension was placed on ice for 5 min and then diluted 10-fold in complete preparation buffer. Following centrifugation, the pellet containing intact nuclei was extracted with either nuclear lysis buffer
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IR induces in vivo dephosphorylation of PP1—We tested if protein Ser/Thr phosphatases were targets of an IR-activated damage-sensing pathway. Jurkat cells were labeled with inorganic 32P for 45 min after irradiation or sham irradiation. Nuclear extracts were prepared and subjected to microcystin affinity chromatography, followed by two-dimensional gel analysis (Fig. 1). Microcystin binds with nanomolar affinity to the catalytic cleft of protein phosphatases (20) and can be used to rapidly and quantitatively recover the various forms of PP1 and PP2A from extracts (21) together with their multiple regulatory subunits (22). On the two-dimensional gel, more than 50 distinct silver-stained proteins were visualized in the elution from the microcystin beads, and about a dozen of these were 32P-labeled. The levels of 32P incorporated into one group of proteins retained by the microcystin matrix dramatically decreased following IR, and we centered our attention on these proteins (Fig. 1). The major form, both by silver staining and 32P labeling, was the protein in spot 3. However, all of these spots were dephosphorylated in response to IR, based on 32P labeling. Tandem mass spectrometric sequencing revealed these proteins were the α and δ isoforms of PP1 (Table I). The differences in migration in the first dimension (isoelectric focusing) for these PP1 isoforms are probably because of formation of intramolecular disulfides and/or differences in oxidation state of the multiple Cys residues in the catalytic subunits. Regardless, the results show IR caused loss of 32P indicating that PP1 is phosphorylated in living cells and IR causes net dephosphorylation.

Effects of IR on Nuclear PP1 in Jurkat and AT Cells—PP1 α and δ isoforms contain a C-terminal RP(I/V)TPPR motif known to be a preferred site for Cdk/Cdc phosphorylation (23). Phosphorylation of the threonine in this motif occurs in yeast as well as mammalian PP1 (24) and attenuates PP1 activity. We and others (25, 26) have shown that IR inhibits Cdk2/cyclin A and Cdk2/cyclin E activity, and both of these kinases phosphorylate PP1α at Thr-320. We reasoned that DNA damage might activate PP1 through reduced phosphorylation of this Thr site. Jurkat cells were irradiated and nuclear extracts were assayed for phosphatase activity at various times post-IR (Fig. 2a). There was more than a doubling in PP1 activity over 90 min. In parallel nuclear extracts were subjected to immunoblot analy-
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| Spot | Identified as | Peptide mass (Da) | pI | Sequence | Accession NCBI no. |
|------|---------------|------------------|----|----------|-------------------|
| 1    | PP1C/α        | 37.5             | 5.94 | (16-26) LLEVQGSRPKG | 4506003 |
|      |               |                  |     | (27-36) NVQLTENEIRG   |        |
|      |               |                  |     | (306-317) YQGFSLNPGGR | 4506005 |
|      | PP1C/δ        | 37.2             | 5.84 | (26-35) IYQMTEAERV |        |
|      |               |                  |     | (132-140) IYGFYDECK |        |
|      |               |                  |     | (168-186) IFCCCHGLSPDLQSMR | 4506003 |
|      |               |                  |     | (304-319) YQIGGLNSGRPVTPFR |        |
|      |               |                  |     | (61-74) ICDINGQYDDLR |        |
|      |               |                  |     | (235-246) FLHKHDDLICR |        |
|      |               |                  |     | (306-323) YQGFSGLPGRPITPR |        |
|      |               |                  |     | (111-121) IKYPENFLLR |        |
|      |               |                  |     | (150-167) TFTDCFNCFLPAAIYDEK | 4506005 |
|      |               |                  |     | (246-259) AQVQVEDGYEFFAK |      |
| 2    | PP1C/α        | 37.5             | 5.94 | (75-96) LEYGGFPPESNYLFGLGVD | 4506003 |
|      |               |                  |     | (60-73) ICDINGQYDTLR |        |
|      | PP1C/δ        | 37.5             | 5.94 | (132-140) IYGFYDECK |        |
|      |               |                  |     | (168-186) IFCCCHGLSPDLQSMR | 4506005 |
|      |               |                  |     | (246-260) AQVQVEDGYEFFAK |      |
| 3    | PP1C/α        | 37.5             | 5.94 | (98-110) QSLETICLLAYK | 4506003 |
|      | PP1C/δ        | 37.5             | 5.94 | (111-121) IKYPENFLLR |        |
|      |               |                  |     | (150-167) TFTDCFNCFLPAAIYDEK |        |
|      |               |                  |     | (246-259) AQVQVEDGYEFFAK |      |
| 4    | PP1C/α        | 37.5             | 5.94 | (502x603) (16-26) LLEVQGSRPKG | 4506003 |
|      |               |                  |     | (27-36) NVQLTENEIRG   |        |
|      |               |                  |     | (222-234) GVSFTPGAEVK |        |
|      | PP1C/δ        | 37.5             | 5.94 | (306-323) YQGFSGLPGRPITPR | 4506005 |
|      |               |                  |     | (132-140) IYGFYDECK |        |
|      |               |                  |     | (168-186) IFCCCHGLSPDLQSMR | 4506005 |
|      |               |                  |     | (246-260) AQVQVEDGYEFFAK |      |

Table 1: Tandem mass spectrometry identification of dephosphorylated proteins.

Fig. 2. a, IR activates nuclear PP1. Jurkat cells (circles) were subjected to 10 Gy, and nuclear extracts were prepared at various times post-IR. PP1c was then immunoprecipitated, and its activity was measured using the phosphopeptide substrate KRPITIRR as described under "Materials and Methods." Quantitation (squares) of the optical density of the immunoblot shown in b was measured by Image Quant 5.0 (Molecular Dynamics, Sunnyvale, CA).

Fig. 3. Thr-320 phosphorylation influences PP1 activity. Purified catalytic subunit of rabbit skeletal muscle PP1 was incubated for 30 min at 30 °C with the indicated concentrations of purified Cdk2 kinase as described under "Materials and Methods." a, an aliquot of the reaction mixture was subjected to immunoblot analysis with phospho-PP1c/α/Thr-320 antibody and anti-PP1c. Quantitation (squares) of the optical density of the immunoblot shown in b. b, PP1c was immunoprecipitated from the remaining reaction mixture and its activity was measured as described for Fig. 2a.
To verify that Thr-320 phosphorylation regulates PP1 activity under our assay conditions, purified skeletal muscle PP1 protein was incubated with purified Cdk2 kinase and subjected to immunoprecipitation with anti-PP1c. PP1 activity in the immunoprecipitates was assayed with a phosphopeptide as substrate. As expected, increasing doses of Cdk2 resulted in increased phosphorylation of this site in PP1 (Fig. 3). Phosphorylation of this site in PP1 corresponded to decreased PP1 activity (Fig. 3b). Thus, in vitro and in living cells the level of Thr-320 phosphorylation inversely correlated with the specific activity of PP1.

To establish that IR inhibited the activity of the endogenous Cdk2 kinase that was phosphorylating PP1, Cdk2 was immunoprecipitated at various intervals after 10 Gy. As shown in Fig. 4, the activity of endogenous Cdk2 toward PP1 at the Thr-320 site decreased in a time-dependent manner following IR.

**IR Both Decreases Cdk2 Kinase and Increases PP1 Phosphatase Activity in an ATM-dependent Manner**—We previously found that IR-induced dephosphorylation of histone H1 is ATM-dependent (14). This is because of reduced Cdk2 kinase and increased nuclear phosphatase activity. IR (10 Gy) inhibits Cdk2 activity in an ATM-dependent manner as shown in Fig. 5a. Cdk2 from AT (PEBS) cells lacking active ATM kinase was inhibited only 40% whereas Cdk2 from reconstituted AT cells (YZ-5) that express ATM was inhibited by 84%. The radiation-induced dephosphorylation of PP1 Thr-320 was ATM-dependent. We compared AT cells (PEBS) and AT cells transfected with full-length ATM (YZ-5) that were irradiated with a dose of 10 Gy. Nuclear extracts were subjected to immunoblot analysis that showed IR failed to dephosphorylate PP1 Thr-320 in AT cells (Fig. 5b). However, in AT cells transfected with full-length ATM, IR-induced dephosphorylation of Thr-320 occurred in a time-dependent manner starting ~15 min post-irradiation. This genetic system was used to demonstrate that IR-induced dephosphorylation of the Thr-320 site of PP1 is dependent on ATM.

The ATM kinase is also necessary for the IR-induced increase in PP1 activity. Nuclear extracts were prepared from AT cells deficient in ATM and AT cells expressing ectopic full-length ATM. Samples were subjected to immunoprecipitation with anti-PP1c and assayed for PP1 activity. As shown in Fig. 5c, the IR-induced time-dependent 2-fold increase in PP1 activity was only observed in cells expressing ATM. Interestingly, the time course of IR activation of PP1 parallels that of IR-induced H1 dephosphorylation, which we have previously reported (14).

Three genes code for four distinct isoforms of PP1 in mammals called α, γ1, γ2, and δ (3). Using isoform-specific antibodies to immunoprecipitate PP1c α and δ from cell lysates, we tested if the Thr-320 site was dephosphorylated in response to IR in Jurkat, AT, and reconstituted AT cells. The numbering of the Thr-320 is not identical in α and δ but lies in the corresponding sequence motif in both these PP1 isoforms. Consistent with the metabolic 32P-labeling results (Fig. 1), both the α and δ PP1 isoforms were dephosphorylated at the Thr-320 site in response to IR (Fig. 6a). Furthermore, the dephosphorylation of both the α and δ isoforms was ATM-dependent, as shown in Fig. 5.
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Fig. 6. IR-induced dephosphorylation of PP1 α and PP1 δ is ATM-dependent. Jurkat (a) and AT fibroblasts PEBS and YZ-5 (b) were irradiated with 10 Gy. Nuclear extracts were prepared and subjected to immunoprecipitation with the indicated isoform-specific anti-PP1c antibodies. Immunoblot analysis was performed as described for Fig. 2. IP, immunoprecipitate.

shown in Fig. 6b. Decreased phosphospecific staining of PP1 α and δ was only seen in irradiated cells that were expressing ATM (YZ-5 clone). Cells that did not express ATM (PEBS) did not show diminished phospho-Thr-320 staining in response to IR.

DISCUSSION

IR is known to activate the G1, S, and G2 checkpoints (2, 27) in an ATM-dependent manner. The ATM gene is mutated in the autosomal recessive disease ataxia telangiectasia, characterized by neuronal degeneration resulting in ataxia, ocullouteraneous telangiectasia, immune dysfunction, and cancer predisposition (28). ATM is thought to be an upstream sensor of DNA damage as well as oxidative stress. ATM transmits the damage signal downstream through its C-terminal phosphoinositol 3-kinase domain. ATM has been shown to directly phosphorylate several proteins and to enhance the phosphorylation of other proteins by activating downstream protein kinases. These targets include the nuclear tyrosine kinase c-Abl, Chk2, nibrin, p53, and BRCA1, all of which have been implicated in DNA damage responses (29–33). Interestingly, ATM has also been shown to control the damage-induced dephosphorylation of Ser-376 in p53 (34) and to regulate H1 dephosphorylation following IR. Thus, ATM activates the phosphatase(s) responsible for dephosphorylating H1 and p53. We have recently demonstrated that IR causes dissociation of the B55α regulatory subunit, which has been implicated in mitotic progression, from heterotrimeric nuclear PP2A in an ATM-dependent manner (38). Although the functional significance of IR-induced dissociation of B55α from PP2A heterotrimer is unknown, it is likely that IR-induced subunit exchange allows PP2A to execute diverse functions in the damage response. PP1 and PP2A are both downstream effectors of an IR-activated and ATM-dependent signaling pathway. It seems likely that IR-induced PP1 activation as well as PP2A subunit exchange requires the function of the kinase domain of ATM, but this remains to be determined.

We imagine two potential but not mutually exclusive mechanisms by which ATM could regulate PP1 activity. First, ATM is known to inhibit Cdc2 and Cdk2 in response to IR. The PP1 Thr-320 site is phosphorylated by Cdc2/Cdk2. Thus, IR-activated ATM could increase PP1 activity indirectly through inactivation of Cdc2/Cdk2 kinase, thereby decreasing the phosphorylation of Thr-320. Because ATM has recently been shown to regulate Cdc25A (the activator of Cdk2) through Chk2, it is possible that ATM regulates PP1 through the Chk2-Cdc25A pathway. If this were the case, then a dual specificity phosphatase (Cdc25) would be regulating another phosphatase (PP1) via a phosphatase-kinase-phosphatase cascade (Cdc25-Cdk2-PP1). Our data do not establish that Cdk2 is the in vivo kinase of PP1. There may be other kinases that phosphorylate the Thr-320 site in PP1, but they would also need to be regulated in response to IR by an ATM-dependent pathway. Alternatively, ATM could directly (or indirectly) phosphorylate a nuclear PP1 subunit that restrains PP1 in its phospholylated, low activity form. Phosphorylation would release inhibition and allow autodephosphorylation of Thr-320 and activation of PP1 toward other substrates. NIPP1 is one example of a nuclear PP1 regulatory subunit whose activity is regulated by phosphorylation (35, 36).

What are the downstream nuclear substrates of PP1 that may be critical in the damage response? Unlike protein serine/threonine kinases, PP1 catalytic subunit does not manifest a high degree of sequence specificity and dephosphorylates multiple substrates. The substrate specificity of PP1 is thought to be modulated through the formation of heterodimeric complexes with regulatory subunits. Regulatory proteins that target PP1 to its substrates in response to DNA damage are not known, and these may bind both α and δ isoforms or there may be individual regulatory subunits for these isoforms. Specific PP1 isoforms have been shown to have distinct functions and locations. For example, the PP1α isoform has been shown to regulate the G1/S transition by dephosphorylating Rb (12), and the PP1β isoform is chromatin-associated. Because histone H1 and histone H3 have been implicated as PP1 substrates (14, 16, 37), it is likely that the PP1β isoform may regulate IR-induced H1 and H3 dephosphorylation.

In summary, the PP1 α and δ isoforms are dynamically phosphorylated in Jurkat cells and in response to IR become activated by dephosphorylation of Thr-320 to function as a downstream effector of an ATM-dependent damage-sensing pathway. Regardless of the mechanism by which ATM activates PP1, defining the regulatory subunits that target PP1 to its respective substrates may reveal novel targets for chemo and radiation sensitizers. Drugs that function at the level of these targets may have low toxicity and, therefore, be more efficacious than less specific inhibitors.

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