Regulation of DNA-dependent Protein Kinase Activity by Ionizing Radiation-activated Abl Kinase Is an ATM-dependent Process*

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Ionizing radiation (IR) treatment results in activation of the nonreceptor tyrosine kinase c-Abl because of phosphorylation by ATM. In vitro evidence indicates that DNA-dependent protein kinase (DNA-PK) also phosphorylate and thus potentially activate Abl kinase activity in response to IR exposure. To unravel the role of ATM and DNA-PK in the activation of Abl, we assayed Abl, ATM, and DNA-PK activity in ATM- and DNA-PKcs-deficient cells after irradiation. Our results show that despite the presence of higher than normal levels of DNA-PK kinase activity, c-Abl fails to become activated after IR exposure in ATM-deficient cells. Conversely, normal activation of both ATM and c-Abl occurs in DNA-PKcs-deficient cells, indicating that ATM but not DNA-PK is required for activation of Abl in response to IR treatment. Moreover, activation of Abl kinase activity by IR correlates well with activation of ATM activity in all phases of the cell cycle. These results indicate that ATM is primarily responsible for activation of Abl in response to IR exposure in a cell cycle-independent fashion. Examination of DNA-PK activity in response to IR treatment in Abl-deficient cells expressing mutant forms of Abl or in normal cells exposed to an inhibitor of Abl suggests an in vivo role for Abl in the down-regulation of DNA-PK activity. Collectively, these results suggest a convergence of the ATM and DNA-PK pathways in the cellular response to IR through c-Abl kinase.

Ionizing radiation (IR) exposure results in DNA damage involving both single and double strand breaks. Such genotoxic events activate a number of signaling pathways that serve, for example, to activate DNA repair mechanisms, halt cell cycle progression, and/or trigger advancement into apoptosis. Intensive efforts focused on elucidating the molecular nature of these pathways have identified many factors that participate in IR-induced DNA damage response. Among those are the c-Abl protein-tyrosine kinase and two members of the phosphatidylinositol 3-kinase family, ATM (ataxia telangiectasia mutated) and DNA-PK.

The proto-oncogene c-Abl encodes a nonreceptor tyrosine kinase that is ubiquitously expressed and localized both in nucleus and cytoplasm (1, 2). c-Abl is required for normal organ development in mice (3). Interestingly, mice expressing a C-terminal truncated Abl also display similar phenotypes (4), indicating that the C terminus of Abl is indispensable for its function. Several functional domains have been identified including a motif that interacts with the C-terminal domain (CTD) of RNA polymerase II, a known substrate for c-Abl (5).

Several studies have shown that the kinase activity of Abl is up-regulated following exposure to IR or other genotoxic agents (e.g. cisplatin, methyl methane sulfonate, mitomycin C and hydrogen peroxide) (1, 6, 7) but not UV light (8). Recent work has established that IR-induced activation of Abl requires ATM function (9, 10). Using yeast two-hybrid and immunoprecipitation approaches, Shafman and co-workers (10) showed that ATM directly interacts with Abl and that activation of Abl kinase activity by IR requires ATM. Following irradiation, ATM kinase phosphorylates Abl on a serine residue located within the kinase domain of Abl resulting in activation of Abl kinase activity (9). These findings clearly indicate the role of ATM as a key regulator of Abl catalytic activity in response to γ irradiation exposure.

Similar to ATM, DNA-PK activity is dramatically up-regulated following the introduction of double strand breaks into the genome. DNA-PK activity is governed by the recruitment of the catalytic subunit of the DNA-PK (DNA-PKcs) to DNA ends through the binding component Ku70/80 complex (11). The importance of DNA-PK activity in genome damage response is exemplified by the extreme radiosensitivity of cells from SCID mice (mice with severe combined immunodeficiency), which are defective in this activity because of a mutation in the DNA-PKcs gene (12, 13). In vitro, DNA-PKcs is capable of phosphorylating and consequently activating Abl, and cells from SCID mice showed reduced activation of Abl kinase activity after IR exposure (18). These observations suggest that DNA-PKcs, like ATM, may also be an upstream regulator of the Abl kinase in response to IR-induced genome damaging events.

We have investigated the physiological relevance of both ATM and DNA-PK activity in the activation of Abl kinase activity following IR exposure. We found that despite the pres-
ence of robust DNA-PK activity in irradiated A-T cells, IR does not activate Abl in this cell type. Conversely, activation of both ATM and Abl occur normally in DNA-PKcs-nullizygous cells. These results indicate that ATM, but not DNA-PK, is involved in the in vivo activation of Abl kinase following IR exposure. Furthermore, IR-dependent activation of both ATM and Abl occur coordinately throughout the cell cycle, suggesting that ATM is the principal regulator of Abl activity. Lastly, evidence suggesting a putative physiological role of Abl in the down-regulation of IR-induced DNA-PK activity mediated by ATM is presented. Together, these results shed some light on the intricate network of kinases associated with the response to IR in vivo.

MATERIALS AND METHODS

Cell Culture and Irradiation—The murine cell line NIH-3T3 and mouse embryonic fibroblasts (MEFs) derived from wild type and DNA-PK-deficient mice (14) were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum. MEFs were used at passage 3. Normal (GM0536A and B-310) and A-T lymphoblastoid (GM012525E and IARC12/AT3) cell lines were grown in RPMI 1640 containing 20% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 × 10−5 M l-glutamine grown in a humidified 5% CO2 atmosphere at 37 °C. The GM0536A and GM012525E lines were obtained from NIGMS Human Genetic Mutant Cell Repository (Camen, NJ). γ irradiation was performed at room temperature using a Gammacell 1000 irradiator (Atomic Energy of Canada, Ltd) with a 137Cs source at a dose rate of 0.21 Gy/min. Following irradiation, cells were returned to the incubator and harvested at the indicated time points.

Antibodies—DNA-PK antibody was purchased from NeoMarkers (San Jose, CA), and ATM antibody was obtained from GenTex (San Antonio, TX). Anti-Abl (8E9) was obtained from Pharmingen (San Diego, CA). Anti-FLAG M2 antibody was obtained from Eastman Kodak Co.

Cell Synchronization—The human lymphoblastoid cell line (GM0536A) was used for the enrichment of cell cycle-specific stages by centrifugal elutriation as described previously (15). Following separation, cell fractions were placed on ice to prevent further cell cycle progression, and cell viability was determined by trypan blue exclusion. Cell cycle distribution of the elutriated fractions was determined by flow cytometry as described (15).

Purification of GST Fusion Proteins—Derivatives of the bacterial expression plasmid pGEX2T containing either human p53 or mouse c-Ab1 (7, 8). This expression vector was pGEX2T containing human p53 (GST-p53) as the substrate (Fig. 1A, upper panel). To confirm that equivalent amounts of DNA-PKcs were present in the kinase assays, immunoprecipitations were assayed for DNA-PKcs levels by immunoblotting (Fig. 1A, lower panel). Consistent with several previous reports (11, 19, 20), a basal level of DNA-PK activity was observed in unirradiated cells. In response to IR, a quantitative increase in DNA-PKcs-associated kinase activity that reached a maximum of approximately 3-fold at 120 min post-IR was observed in both normal (Fig. 1A, lanes 1–4) and A-T lymphoblasts (Fig. 1A, lanes 5–8).

Although the overall kinetics of irradiation-induced DNA-PK activity were similar between normal and A-T cells (Fig. 1B), a quantitatively higher (1.6-fold ± 0.3, n = 3) level of DNA-PK activity was consistently observed at 60 and 120 min post irradiation in A-T lymphoblasts when compared with wild type controls. In addition, at 4 and 8 h post irradiation the DNA-PK activity in normal cells returned to basal level, whereas that of A-T cells remained at least 1.8-fold higher than basal level (data not shown). Taken together, these results indicate that A-T cells exhibit enhanced levels of IR-induced DNA-PK activity and lack of down-regulation of DNA-PK activity.

As previously observed, irradiation of normal cells leads to a rapid increase in Abl-associated kinase activity as judged by the in vitro phosphorylation of a recombinant protein encoding the C-terminal domain of DNA polymerase II (GST-CTD) (Fig. 1C, lanes 1 and 2). However, despite the documented presence of robust DNA-PK activity in A-T cells (Fig. 1A), activation of Abl kinase as a consequence of irradiation was not observed in this cell type (Fig. 1C, lanes 3 and 4). These results clearly indicate that DNA-PK activation after IR irradiation is dispensable for the in vivo activation of Abl.

Activation of Abl and ATM Is Normal in DNA-PKcs-deficient Cells—To evaluate the requirement of DNA-PK activity in IR-induced activation of Abl kinase activity, we assayed Abl and ATM kinase assays performed as described previously (8, 9).

RESULTS

A-T Cells Display IR-induced Activation of DNA-PK but Not c-Ab1 Activity—Ionizing radiation treatment results in activation of tyrosine kinase activity of c-Ab1 (7, 8). This IR-mediated Abl kinase activation requires ATM, because cells deficient in ATM fail to activate Abl (9, 10). In addition to ATM, another member of the phosphatidylinositol 3-kinase family, DNA-PKcs, has also been shown to activate Abl kinase in vitro (18), prompting us to test the in vivo activation of DNA-PK and Abl as physiological responses to irradiation in normal and A-T cells.

Normal and A-T lymphoblastoid cells were exposed to 5 Gy of γ radiation and DNA-PK activity was determined by an in vitro kinase reaction using recombinant human p53 (GST-p53) as the substrate (Fig. 1A, upper panel). To confirm that equivalent amounts of DNA-PKcs were present in the kinase assays, immunoprecipitations were assayed for DNA-PKcs levels by immunoblotting (Fig. 1A, lower panel). Consistent with several previous reports (11, 19, 20), a basal level of DNA-PK activity was observed in unirradiated cells. In response to IR, a quantitative increase in DNA-PKcs-associated kinase activity that reached a maximum of approximately 3-fold at 120 min post-IR was observed in both normal (Fig. 1A, lanes 1–4) and A-T lymphoblasts (Fig. 1A, lanes 5–8).

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Activation of Abl and ATM Is Normal in DNA-PKcs-deficient Cells—To evaluate the requirement of DNA-PK activity in IR-induced activation of Abl kinase activity, we assayed Abl and ATM kinase assays performed as described previously (8, 9).
DNA-PKcs null mice (14). MEFs isolated from DNA-PK−/− and wild type controls were exposed to 5 Gy of γ radiation, and Abl activity was quantitatively assessed by an in vitro kinase assay. Activation levels of Abl kinase activity after IR exposure were approximately 3.5-fold higher than unexposed control (Fig. 2A, upper panel) in wild type (Fig. 2A, lanes 1 and 2) and DNA-PK−/− MEFs (Fig. 2A, lanes 3 and 4). This result indicates that irradiation-induced Abl activation is normal in the absence of DNA-PK activity.

To examine whether irradiation-induced Abl activation correlates with ATM activation, we measured ATM kinase activity in DNA-PK−/− and wild type MEFs at various times after exposure to γ irradiation (Fig. 2B). Quantitative analysis of three independent experiments indicates comparable kinetics of ATM kinase activation after IR exposure in both the cell types (Fig. 2B and C). Taken together, these findings clearly indicate that coordinated activation of ATM and Abl kinases occurs normally in DNA-PK-deficient cells.

**Activation of ATM and Abl during the Cell Cycle**—Several studies have shown that the induction of Abl kinase activity is under cell cycle regulation (21). For example, in response to the DNA alkylating agent methyl methane sulfonate, activation of Abl kinase is observed in both G1 and S phase but not in G0 (8). To examine whether activation of ATM and the ATM-dependent activation of Abl are also under cell cycle control, we fractionated logarithmically growing normal lymphoblastoid cells by centrifugal elutriation and subjected various fractionated populations to IR (5 Gy). ATM (Fig. 2A) and Abl (Fig. 2B) kinase activity was measured using recombinant Abl (GST-KD-Abl) and RNA Pol II (GST-CTD) fusion proteins, respectively. In asynchronous (Fig. 2A, lanes 1–4), G1 phase (Fig. 2A, lanes 5–8), and S phase (Fig. 2A, lanes 9–12) cells, low basal levels of ATM activity were observed, and a clearly detectable increase in ATM kinase activity was observed as early as 5 min and was maximally (~3–4-fold) up-regulated at 60 min post irradiation. Fractionated M phase cells exhibited similar ATM kinase activity after irradiation (lanes 13–15) but had a higher basal level of ATM activity in unirradiated control cells. Immunoblot analysis of ATM immunoprecipitates shows that ATM protein levels remained unchanged during the cell cycle as previously reported (22, 23).

Closely reflecting what was observed in the ATM kinase activity outlined above, analysis of Abl activity in asynchronous, G1 and S phase populations showed up-regulation of Abl activity in both G1 and S phase but not in G0 (8).
kinase activity within 15 min with maximal activation of ∼4–5-fold at 60 min after irradiation (Fig. 3B, lanes 1–12). In agreement with previous reports (24, 25), increased basal activity of Abl was observed in mitotic cells. Nevertheless, both ATM and Abl kinase activities are coordinately up-regulated in response to IR exposure in these cell populations. Together, these findings strongly suggest that ATM is capable of regulating Abl activity in response to IR-induced genome damage throughout the cell cycle.

**Abl Negatively Regulates DNA-PK Activity**—c-Abl-dependent tyrosine phosphorylation of DNA-PKcs has been recently reported in response to IR exposure (18). In an attempt to explain the higher level of DNA-PK activity found in irradiated A-T cells, we examined the effect of STI 571 (CGP 57148), an ATP-competitive inhibitor of the Abl protein kinase on DNA-PK activity. The STI 571 compound has been reported to inhibit oncosgenic forms of Abl such as V-Abl, Bcr-Abl as well as c-Abl kinase activity at 1 μM (26). At 10 μM concentration STI 571 strongly inhibited irradiation-induced Abl kinase activity in 3T3 cells expressing wild type Abl (Fig. 4A). In addition, a time course response after irradiation shows similar levels of induction (∼3-fold) of DNA-PK activity in STI 571-treated or untreated cells (Fig. 4B). However, unlike untreated cells, STI-treated cells failed to down-regulate DNA-PK activity after 8 h. These results show that inhibition of irradiation-induced Abl activity results in the loss of down-regulation of DNA-PK activity that normally occurs after irradiation.

To complement the study described above we next examined Abl−/− cells expressing wild type and mutated Abl for their ability to respond to irradiation. Previous studies established that IR-dependent activation of Abl requires the phosphorylation of Ser465 located within the Abl kinase domain, because mutation of Ser465 to alanine (Abl-S465A) renders Abl refractile to IR activation (9). Furthermore, mutational analysis of MAPK has suggested that the replacement of a critical serine residue that serves as a site for phosphorylation to a glutamic acid could mimic the effect of phosphorylation at this site (28). Hence, we reasoned that mutation of Ser465 to glutamic acid (Abl-S465E) should render Abl constitutively active.

We tested Abl-deficient MEFs that have been stably transfected with constructs that express either of these mutants (Abl-S465E and Abl-S465A) or wild type Abl for irradiation-induced Abl kinase activation. Cells expressing wt-Abl show a 4–5-fold enhancement in kinase activity following IR exposure. Consistent with a previous report (9), the S465A mutant did not show activation following irradiation (Fig. 5A, lanes 3 and 4). In contrast, Abl-S465E (Fig. 5A, lanes 5 and 6), displayed a higher (∼4-fold) level of basal activity than unirradiated cells expressing either wt-Abl or Abl-S465A. Further, the increased basal activity of Abl mutant (Abl-S465E) is comparable with the activation levels achieved after 1 h of irradiation in cells expressing wt-Abl (Fig. 5A, compare lanes 2 and 5). Irradiation did not result in notable up-regulation of Abl-S465E activity, suggesting that Ser465 is the primary residue in IR-induced activation of Abl.

Next, we subjected cells expressing wt-Abl, Abl-S465A, Abl-S465E, or vector alone to 5 Gy of IR and assayed their lysates for DNA-PK activity. Consistent with the results observed in normal human lymphoblastoid cells (Fig. 1A), activation of DNA-PK activity was observed in irradiated cells expressing wt-Abl 4 h post-IR. At 8 h we observed a significant down-regulation of DNA-PK activity (Fig. 5B, lanes 1–3) similar to the results shown in Fig. 4B, lanes 1–4. Cells expressing Abl-S465A showed a similar level of IR-induced activation of DNA-PK activity (Fig. 5B, compare lanes 1 and 4); however, at the 8-h time point these cells displayed no decrease in the DNA-PK activity (Fig. 5B, lanes 4–6). A similar result was
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obtained in cells reconstituted with vector control (lanes 10–12). These findings suggest that inactivating the ability of Abl to respond to ATM phosphorylation results in decreased regulation of DNA-PK activity. Conversely, Abl-S465E cells displayed low DNA-PK activity both before and after irradiation (Fig. 5B, lanes 7–9) when compared with Abl-S465A or wt-Abl expressing cells. Taken together, these results indicate that one physiological function of the c-Abl activation by ATM in the response to IR exposure might be to down-regulate DNA-PK activity.

FIG. 5. Alteration of the ATM phosphorylation site on Abl affects DNA-PK activity following irradiation. A, Abl-deficient MEFs stably expressing FLAG-tagged wild type human Abl, S465A, or S465E Abl mutants were exposed to 5 Gy of ionizing radiation and harvested after 1 h. Anti-FLAG was used to immunoprecipitate Abl, and the immune complex was subjected to an in vitro kinase assay using GST-CTD as the substrate (upper panel) or immunoblotted with anti-Abl to confirm equivalent abundance of kinase (lower panel). Relative signal intensity of the radiolabeled GST-CTD is indicated. B, Abl-deficient MEFs expressing wild type Abl or the S465A or S465E mutant forms of Abl or vector alone were exposed to 5 Gy of IR, harvested at the indicated time point, and subjected to an in vitro kinase assay to examine DNA-PK activity using GST-p53 as the substrate (top panel). An aliquot of the immune complex was used to analyze for the amount of DNA-PKcs in each of the kinase reactions by immunoblotting with anti-DNA-PK antibody (bottom panel).

DISCUSSION

In this study we provide evidence that shows that ATM but not DNA-PK activity is required for the in vivo catalytic activation of Abl in response to IR-induced genome damage. In addition, we demonstrate that IR-induced activation of Abl correlates well with activation of ATM in both interphase and mitotic cells. This conclusion is consistent with recent findings (29) that ATM-dependent phosphorylation of p53 occurs in an irradiation-dependent manner throughout the cell cycle.

Despite the fact that DNA-PK is capable of phosphorylating and consequently activating Abl in vitro (18), DNA-PK activity is not required for in vivo activation of Abl kinase in response to γ irradiation. Two lines of evidence support this conclusion. First, A-T cells show normal elevation of DNA-PK activity after irradiation but fail to exhibit Abl activation. Second, Abl activity after IR treatment is comparable between DNA-PKcs-proficient and DNA-PKcs-deficient murine cell lines. Together, these findings lead to the unequivocal conclusion that ATM is an essential regulator of Abl activity after γ irradiation and that DNA-PK does not perform a parallel function in vivo. A previous study (18) reported that c-Abl activity was marginally elevated in irradiated mouse SCID cells. Although the SCID cell lines employed in those studies are DNA-PK defective, they have been adapted in culture by high number of passages during which additional mutations might have accumulated, resulting in compromised Abl activation following IR treatment.

Preliminary evidence demonstrated a physical interaction between DNA-PKcs and c-Abl. In addition, several groups reported that in vitro phosphorylation of DNA-PKcs by Abl, Lyn, or protein kinase Cδ result in dissociation of this subunit from the Ku complex (20, 30). In the same context, the binding of Ku and c-Abl at an overlapping C-terminal region of DNA-PKcs has been proposed as another mechanism for regulating DNA-PK activity (20). In this study we provide evidence that indicates a physiological role for Abl in the down-regulation of DNA-PK activity. We first show that pre-exposure of normal cells to an ATP-competitive inhibitor of the Abl kinase, STI 571, renders normal cell lines unable to down-regulate DNA-PK activity at 8 h post irradiation. Also, the Abl-deficient cell line reconstituted with an IR-refractile mutant Abl (S465A) or vector alone was unable to down-regulate DNA-PK activity after IR. Furthermore, the constitutively active S465E-Abl mutant suppressed IR-dependent induction of DNA-PK activity completely. Together, these results suggest that one of the physiological roles of c-Abl activation in response to IR exposure is to down-regulate the DNA-PK activity. This c-Abl-mediated down-regulation of DNA-PK activity may explain the observed higher level of DNA-PK activity (Fig. 1B), as well as the failure of DNA-PK activity to return to basal levels post irradiation in A-T cells (data not shown).

DNA-PK has been proposed to play an essential role in activating DNA repair mechanisms (11). Studies of asynchronously growing cells suggest that the bulk of the DNA repair following IR exposure occurs within 2–4 h and that the remainder being completed in the subsequent 24–48 h (11, 20). Previous studies have shown that physical interaction with and/or phosphorylation of DNA-PKcs by Abl, Lyn, or protein kinase Cδ results in the dissociation of DNA-PKcs from the Ku70/80 heterodimer (20, 27). The timing of Abl-mediated inactivation of DNA-PK, which we have shown to be 4–8 h post irradiation, suggests that Abl could facilitate release of free Ku70/80 heterodimer from sites of double strand breaks in the slow phase of double strand breaks repair.

It is worth noting that c-Abl and DNA-PK bind to and phosphorylate the C-terminal domain of RNA polymerase II. In agreement with this observation, tyrosine phosphorylation of RNA polymerase II is increased after IR exposure in an ATM/Abl-dependent manner (9). Thus, Abl may regulate transcription in response to IR exposure by phosphorylating RNA polymerase II. In this context, another role of the regulation of DNA-PK activity by c-Abl might be associated transcription modulation after irradiation.

In conclusion, we have demonstrated that ATM, but not DNA-PK activity is required for IR-induced activation of Abl kinase and that this induction occurs in a cell cycle-independent manner. Furthermore, in agreement with the in vitro results reported elsewhere, we show results that suggest an in vivo role of c-Abl in the down-regulation of DNA-PK activity after γ irradiation. Finally, kinase activity encoded by ATM and Abl clearly contribute to the DNA damage response process, at least in part, through regulation of DNA-PK activity.

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