Regulation of Rad51 Function by c-Abl in Response to DNA Damage*

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The Rad51 protein, a homolog of bacterial RecA, functions in DNA double-strand break repair and genetic recombination. Whereas Rad51 catalyzes ATP-dependent pairing and strand exchange between homologous DNA molecules, regulation of this function is unknown. The c-Abl tyrosine kinase is activated by ionizing radiation and certain other DNA-damaging agents. Here we demonstrate that c-Abl interacts constitutively with Rad51. We show that c-Abl phosphorylates Rad51 on Tyr-54 in vitro. The results also show that treatment of cells with ionizing radiation induces c-Abl-dependent phosphorylation of Rad51. Phosphorylation of Rad51 by c-Abl inhibits the binding of Rad51 to DNA and the function of Rad51 in ATP-dependent DNA strand exchange reactions. These findings represent the first demonstration that Rad51 is regulated by phosphorylation and support a functional role for c-Abl in regulating Rad51-dependent recombination in the response to DNA damage.

The c-Abl nonreceptor tyrosine kinase is activated in cells exposed to ionizing radiation (IR) and certain other DNA-damaging agents (1–4). IR induces DNA double-strand breaks (5) and thereby activates the DNA-dependent protein kinase (DNA-PK) (6–8). Recent work has shown that DNA-PK phosphorylates and activates c-Abl (9). Other studies have demonstrated that c-Abl interacts with the ataxia telangiectasia mutated (ATM) gene product and that ATM may activate c-Abl in the response to genotoxic stress (10, 11). Whereas cells deficient in DNA-PK or ATM are hypersensitive to killing by IR (12, 13), c-Abl-deficient cells are resistant to IR-induced apoptosis (14). Activation of c-Abl by genotoxic stress is associated with interaction of c-Abl with the p53 tumor suppressor in the G1 arrest response (15, 16). Other signals dependent on c-Abl activation include induction of the stress-activated protein kinase and p38 mitogen-activated protein kinase by genotoxic agents (1, 2, 17). The findings that c-Abl contributes to the regulation of p53 and certain stress-activated kinases associated with apoptosis have provided support for the activation of c-Abl as a pro-apoptotic signal (14). In this context, expression of c-Abl is associated with G1 phase growth arrest and induction of apoptosis (14, 18, 19).

Recombination plays a fundamental role in the repair of DNA damage. In Escherichia coli, the RecA protein mediates repair of double-strand breaks by initiating pairing and strand exchange between homologous DNAs (20). Identification of structural homologs of RecA in yeast, Xenopus laevis, mouse, and human cells has supported conservation of similar repair functions throughout evolution (21–25). ScRad51, the RecA homolog in Saccharomyces cerevisiae, is required for DNA damage-induced mitotic recombination (21). ScRad51 converts DNA double-strand breaks to recombinational intermediates, and rad51 mutants accumulate these breaks during meiosis (21). The finding that human Rad51 (HsRad51) promotes homologous pairing and strand exchange reactions in vitro has suggested that Rad51 may also play a role in recombinational repair in man (26). Whereas yeast deficient in Rad51 are viable (21), targeted disruption of the rad51 gene in mice results in an embryonic lethal phenotype (27, 28). These findings in rad51−/− mice have suggested that mammalian Rad51 has an essential role in cell proliferation and/or maintenance of genomic stability.

The present studies demonstrate that c-Abl associates with Rad51. We show that c-Abl phosphorylates Rad51 on Tyr-54 in vitro and in irradiated cells. Importantly, phosphorylation of Rad51 by c-Abl inhibits Rad51 function in DNA strand exchange assays.

MATERIALS AND METHODS

Cell Culture—U-937 cells, HeLa cells, 293 embryonal kidney cells, and mouse embryo fibroblasts (Abl−/−, Abl+/−) (29) were grown as described (1). Irradiation was performed using a Gammacell 1000 (Atomic Energy of Canada) with a 137Cs source emitting at a fixed dose of 0.21 gray min−1 as determined by dosimetry. Immunoprecipitations and Immunoblot Analyses—Cell lysates were prepared as described in Iysis buffer containing 0.5% Nonidet P-40 (15). Immunoprecipitations were performed as described (15) with anti-c-Abl (Ab-3, Oncogene Science), anti-human Rad51 (30), anti-Tyr(P) (4G10, Upstate Biotechnology, Inc.) or anti-Flag (M2, Eastman Kodak Co.). Proteins were separated in 8% or 10% SDS-polyacrylamide gels and probed with anti-c-Abl, anti-Flag, anti-Tyr(P), or anti-Rad51.

Fusion Protein Binding Assays—Purified GST, GST-c-Abl (full-length) (31), GST-Ab3 SH3 (32), and GST-GRB2 N-SH3 (33) proteins (5 µg) were incubated with cell lysate or with purified Rad51 (7 µg) for 2 h at 4 °C. The adsorbates were analyzed by immunoblotting with anti-c-Abl, anti-Flag, anti-Tyr(P), or anti-Rad51.

In Vitro Kinase Assays—Kinase-active c-Abl purified from baculovirus was incubated with purified HsRad51, GST-IXo or GST-Crk, and 125I-PATP in kinase buffer (20 mM HEPES, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl2) for 15 min at 30 °C. Purified c-Src (14–117, Upstate Biotechnology, Inc.) and enolase (Sigma) as a substrate were included as controls. Phosphorylated proteins were separated by 10% SDS-PAGE and analyzed by autoradiography.

Expression of c-Abl, c-Abl(K-R), and Flag-HsRad51—A vector expressing Flag-HsRad51 was generated by subcloning polymerase chain

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The abbreviations used are: IR, ionizing radiation; DNA-PK, DNA-dependent protein kinase; ATM, ataxia telangiectasia mutated; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; dsDNA, double-strand DNA; ssDNA, single-strand DNA; PAP, potato acid phosphatase.
reaction-generated HsRad51 into a Flag-tag pcDNA3 vector. The vector was stably expressed in HeLa cells by selection in the presence of neomycin. Alternatively, Flag-HaRad51 was transiently cotransfected with c-Abl or c-Abl(K-R) vectors (15, 18) into 293 cells by the calcium phosphate method. The cells were harvested 48 h post-transfection and lysed in Nonidet P-40 lysis buffer.

**Mass Spectrometry—**HsRad51 was separated by 8% SDS-PAGE and identified by Copper Stain (Bio-Rad). The protein band was excised, destained, and digested with trypsin (Boehringer Mannheim). The tryptic peptides were extracted and analyzed by matrix-assisted laser desorption/ionization mass spectrometry.

**Strand Exchange Reactions—**ScRad51 (18 µg; 0.42 nmol) was incubated with kinase-active or heat-inactivated c-Abl in 10 µl of buffer A (42 mM potassium MOPS, pH 7.2, containing 1 mM dithiothreitol, 80 mM KCl, 3 mM MgCl₂, 2.5 mM ATP) for 5 min at 37 °C. DNA viral (+) strand (390 ng; 1.2 nmol of nucleotide) was added for 7 min at 37 °C. Single-strand DNA-binding protein (3.8 µg; 50.3 pmol) was then added for an additional 7 min. After incorporation of 390 ng of double-strand dX 174 DNA (linearized with PstI; 1.2 nmol nucleotides) in 3 µl of 50 mM spermidine, the reaction mixture (37.5 µl) was incubated at 37 °C. Aliquots were processed as described (34) and analyzed in 0.8% agarose gels.

**Preparation of His-tagged HsRad51—**A vector expressing His tag HsRad51 was prepared by cloning polymerase chain reaction-generated HsRad51 into the pET-28a-c(+) vector (Novagen). The identity of the subcloned HsRad51 was confirmed by restriction enzyme digestions and DNA sequencing. His tag HsRad51 was expressed in E. coli and purified with His-BIND Resin (Novagen). Phosphorylated His tag HsRad51 was obtained by incubating the resin-conjugated HsRad51 with excess purified c-Abl in kinase buffer containing ATP at 30 °C for 30 min. After washing, the phosphorylated HsRad51 was incubated in the presence and absence of potato acid phosphatase (Sigma) and then eluted from the resin. The final products were dialyzed and concentrated by ultrafiltration.

DNA Binding Assays—**Kinase-active c-Abl (1 µg; 68.9 pmol) and/or Rad51 (6 µg; 0.14 nmol) were preincubated in buffer A for 5 min. dX 174 viral ssDNA (130 ng; 0.4 nmol) and 100 µg/ml bovine serum albumin were then added to the reaction mixture and incubated at 37 °C for an additional 10 min. Samples were analyzed in 0.8% agarose gels.

**RESULTS AND DISCUSSION**

Whereas IR induces DNA double-strand breaks and activates c-Abl (1), we asked if c-Abl interacts with proteins, such as Rad51, that are involved in DNA double-strand break repair. Analysis of anti-c-Abl immunoprecipitates with an anti-Rad51 antibody demonstrated reactivity with a 38-kDa protein (Fig. 1A). When anti-Rad51 immunoprecipitates were analyzed by immunoblotting with anti-c-Abl in the reciprocal experiment, the results confirmed a constitutive association of c-Abl and human (Hs) Rad51 (Fig. 1A). The interaction between c-Abl and HsRad51 was unaffected by irradiation of the cells (data not shown). Also, the finding that DNase has no effect on the coimmunoprecipitation of c-Abl and HsRad51 indicated that the association between these proteins is not dependent on DNA binding (data not shown). To assess the interaction of c-Abl and HsRad51 in vitro, we incubated GST fusion proteins prepared from c-Abl with U-937 cell lysates. Analysis of the adsorbates with anti-Rad51 demonstrated binding between c-Abl and HsRad51 (Fig. 1B). Similar experiments with GST-Abl SH3 but not with a GST fusion protein derived from the N-terminal domain of Grb2, revealed binding to HsRad51 (Fig. 1B). To determine whether the interaction between c-Abl and HsRad51 is direct, we incubated GST-Abl SH3 with purified HsRad51. Immunoblot analysis of the adsorbate with anti-Rad51 demonstrated direct binding of c-Abl and HsRad51 (Fig. 1B, last lane). The c-Abl SH3 domains binds to a consensus proline-rich PXXP sequence (32, 35). The presence of two PXXP
sites in HsRad51 (amino acids 283–286 and 318–321) provides further support for a direct interaction between the c-Abl SH3 domain and the C-terminal region of HsRad51.

To determine whether c-Abl phosphorylates Rad51, we incubated recombinant c-Abl with purified HsRad51 in the presence of [32P]ATP. Analysis of the products by autoradiography indicated that HsRad51 is a substrate for c-Abl (Fig. 2A, left) and not for c-Src (Fig. 2A, right). Similar results were obtained with S. cerevisiae (Sc) Rad51 (data not shown). To assess whether Rad51 is phosphorylated on tyrosine in vivo, we studied HeLa cells that stably express Flag-tagged HsRad51 (HeLa/Rad51) (Fig. 2B, right). The HeLa/Rad51 cells were transfected to express the dominant negative c-Abl(K-R) mutant or wild-type c-Abl (Fig. 2B). Analysis of anti-c-Abl immunoprecipitates for phosphorylation of GST-Crk (1) demonstrated that IR-induced activation of c-Abl is blocked in cells expressing c-Abl(K-R) (Fig. 2C, left). To assess tyrosine phosphorylation of Rad51, anti-Flag immunoprecipitates were subjected to immunoblotting with anti-Tyr(P). Whereas there was little if any reactivity of Flag-tagged HsRad51 with anti-Tyr(P) in irradiated cells expressing c-Abl(K-R), IR exposure induced tyrosine phosphorylation of HsRad51 in the presence of wild-type c-Abl (Fig. 2C, right). These findings provided support for IR-induced tyrosine phosphorylation of Rad51 by a c-Abl-dependent mechanism.

To confirm the in vivo interaction between c-Abl and Rad51, 293 cells were transfected to transiently express both Flag-tagged HsRad51 and kinase-active c-Abl or kinase-inactive c-Abl(K-R) (15) (Fig. 3A). Lysates from the transfec}
c-Abl Regulates Rad51 Activity

HeSrad51 forms a complex with c-Abl that includes ATM (data not shown). Therefore, the available evidence supports a model in which activation of c-Abl by DNA damage down-regulates Rad51 activity and thereby recombination repair. c-Abl could also affect an essential function of Rad51 that involves interactions with other proteins such as p53 (38) or BRCA1 (39). Whereas cells deficient in c-Abl are resistant to the lethal effects of IR (14) and other DNA-damaging agents (40), c-Abl may regulate repair of DNA lesions. Also, the demonstration that yeast cells deficient in Rad51 are defective in DNA double-strand break repair (21) and that HeSrad51 promotes DNA strand exchange in vitro (26) has supported a role for Rad51 in recombinatorial repair. These findings, together with the demonstration that c-Abl regulates Rad51, suggest that activation of c-Abl by IR (1) and thereby inhibition of Rad51 function could determine cell fate by controlling DNA repair and consequently cell survival.

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