c-Abl Tyrosine Kinase Is Not Essential for Ataxia Telangiectasia Mutated Functions in Chromosomal Maintenance*

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Noriaki Takao, Ryoichi Mori, Hideaki Kato‡, Akira Shinohara§, and Ken-ichi Yamamoto¶

From the Department of Molecular Pathology, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan and the ¶Department of Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois 60637

c-Abl is activated by DNA damage in an ataxia telangiectasia mutated (ATM)-dependent manner and plays important roles in growth arrest and apoptosis induced by DNA damage. c-Abl also interacts physically and functionally with Rad51, a key molecule in homologous recombinational (HR) DNA repair. To study further the roles of c-Abl in HR DNA repair, we generated c-Abl−/− and ATM−/−/c-Abl−/− mutant cell lines from a chicken B lymphocyte DT40 cell line, comparing the phenotypes of these mutants to those of ATM−/−/c-Abl−/− DT40 cells that we had created previously. We found that the time course of radiation-induced Rad51 focus formation is abnormal in ATM−/−/c-Abl−/− DT40 cells, consistent with the observation that ATM−/−/c-Abl−/− DT40 cells display hypersensitivity to ionizing radiation and highly elevated frequencies of both spontaneous and radiation-induced chromosomal aberrations. In contrast, c-Abl−/− cells did not show these ATM-related defects in their cellular response to radiation, nor did the disruption of c-Abl in ATM−/− DT40 cells exacerbate these ATM-related defects. However, c-Abl−/−/c-Abl−/− DT40 cells, but not ATM−/−/c-Abl−/− DT40 cells, were resistant to radiation-induced apoptosis, indicating an important role for c-Abl in this cellular response to ionizing radiation. These results therefore indicate that, although ATM plays an important role in genome maintenance, c-Abl is not essential for this ATM function. These findings suggest that c-Abl and ATM play important roles in the maintenance of the cell homeostasis in response to DNA damage that are, at least in part, independent.

Ataxia telangiectasia (A-T), 1 caused by mutations in the ATM gene, is a recessive chromosomal instability disease with pleiotropic clinical phenotypes involving the nervous, immune, and reproductive systems. A predisposition to lymphoid malignancy and extreme radiosensitivity are other features of the disease. Cells derived from A-T patients are characterized by a high level of chromosomal abnormalities, hypersensitivity to ionizing radiation (IR), and defective cell cycle regulation, suggesting that these cellular anomalies result from a disruption of the ATM gene. ATM is a member of the family of large proteins carrying a carboxyl-terminal phosphatidylinositol 3-kinase-like domain (1, 2); this family includes Saccharomyces cerevisiae Mec1, Tor1, and Tor2, Saccharomyces pombe Rad3, Drosophila melanogaster Mei-41, human ATR, and the catalytic subunit of the vertebrate DNA-dependent protein kinase, all of which have roles in cell cycle checkpoint control and/or DNA repair (3, 4).

Although the precise functions of ATM in the cellular reaction to DNA damage have remained unclear, recent work has shown ATM to directly bind (5) and phosphorylate (6) c-Abl, one of key effectors of this reaction. c-Abl, first identified as the cellular homologue of v-Abl from the Abelson murine leukemia virus (7), is ubiquitously expressed and shares certain structural features with the Src family of tyrosine kinases (8). c-Abl binds p53, and over-expression of c-Abl is associated with growth arrest in the G1 phase by p53-dependent mechanisms (9, 10). Other studies have shown that c-Abl-deficient cells exhibit resistance to apoptosis induced by IR, and over-expression of c-Abl induces apoptosis (11). More recent studies have demonstrated that c-Abl activates the p53-related p73 in the apoptotic response to DNA damage (12, 13). The results of these studies indicate, therefore, that c-Abl plays important roles in growth arrest and the apoptosis induced by DNA damage. Other signals dependent on c-Abl activation include induction of the stress-activated protein kinase and the p38 mitogen-activated protein kinase (14, 15).

Homologous recombinational (HR) DNA repair plays a fundamental role in genome maintenance following DNA damage. Two groups of investigators have recently demonstrated functional interactions of c-Abl with Rad51, which is a eukaryotic homologue of the bacterial recombinase RecA and plays a key role in HR DNA repair (16, 17). One study showed that c-Abl inhibits binding of Rad51 to DNA by phosphorylating it on tyrosine 54, indicating that c-Abl negatively regulates Rad51 to function as a recombinase (18). The other study showed that c-Abl phosphorylates Rad51 on tyrosine 315 and that this phosphorylation enhances the association of Rad51 with Rad52 (19), although a possible significance of this c-Abl-dependent Rad51/Rad52 interaction in the Rad51 recombinase function remains obscure. To further study the roles of c-Abl in HR DNA repair, we generated c-Abl−/− and ATM−/−/c-Abl−/− mutant clones from DT40 cells and compared the phenotypes of these mutant DT40 cells with those of ATM−/− mutant DT40 cells that we had created recently (20). The results presented in this study indicate that, whereas ATM functions are essential for genome maintenance, as has been shown previously, ATM does not require c-Abl for its functions in DNA maintenance.

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¶ To whom correspondence should be addressed: Dept. of Molecular Pathology, Cancer Research Institute, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-0934, Japan. Tel.: 81-76-265-2755; Fax: 81-76-234-4516; E-mail: kyamamoto@kenroku.kanazawa-u.ac.jp.
§ The abbreviations used are: A-T, ataxia telangiectasia; ATM, ataxia telangiectasia mutated; ATR, ATM-related; DSB, double-stranded break; FACS, fluorescence-activated cell sorting; HR, homologous recombination; IR, ionizing radiation; RT-PCR, reverse transcriptase-polymerase chain reaction.
EXPERIMENTAL PROCEDURES

Construction of Targeting Vectors—A partial chicken c-Abl cDNA was obtained by RT-PCR with degenerative primers (5′-GGACACCAT-GGAGGGGAAAG-3′ and 5′-GGRTTCCAYTGCARANGC-3′) using mRNA extracted from DT40 cells. Using this cDNA fragment as a probe, chicken c-Abl genomic clones were isolated by screening an EMBL3 SP6/T7 genomic library made from the liver of an adult male Leghorn chicken (CLONTECH, Palo Alto, CA) using standard procedures. As shown in Fig. 1A, c-Abl disruption constructs were made by replacing about 500 base pairs of the genomic sequence of the chicken c-Abl kinase region with selection marker gene cassettes under the control of the β-actin promoter.

Cell Culture and Gene Targeting—The cell culture conditions and DNA transfection procedures were as described previously (20). To generate c-Abl−/− mutant clones, one allele was disrupted by selection in medium containing 1 mg/ml histidinol (Sigma) after transfecting wild-type DT40 cells with pAABL-his, and the other allele was disrupted by transfecting the c-Abl−/− mutant clones with pAABL-puro, followed by selection with both histidinol and 0.5 μg/ml puromycin (Sigma). To generate ATM−/−/c-Abl−/− double mutant clones, ATM−/− DT40 cells (20) were sequentially transfected with c-Abl-targeting constructs containing histidinol or blasticidin-S resistant gene cassettes. Cells were then selected in medium containing histidinol after the first transfection and then both histidinol and 25 μg/ml blasticidin-S (Calbiochem) after the second transfection. RT-PCR analysis of chicken c-Abl and ATM mRNA expression was performed as described previously (20), using the following primers: ATM, 5′-GTTGGATCCTAGGCAGAGAT-3′ and 5′-GTGAGCTTCATCCTCTGTC-3′; c-Abl, 5′-GAGGACACC-ATGAGGGGAAGACTTGCTTG-3′ and 5′-GGAGACCTTGTTGTTGAGCCAGGCTCTTGG-3′.

Immunofluorescent Visualization of Rad51 Foci—Rad51 foci were visualized using confocal microscopy (MRC-1024, Bio-Rad) of cells stained with an anti-Rad51 antibody as described previously (21). Unvisualized using confocal microscopy (MRC-1024, Bio-Rad) of cells transfected with the probe shown in panel A, C, RT-PCR analysis of c-Abl mRNA expression in wild-type and various mutant DT40 cells.

RESULTS AND DISCUSSION

For targeted disruption of c-Abl, targeting vectors (pAABL-his and pAABL-puro) were constructed by inserting selection-drug resistance gene cassettes into the c-Abl genomic sequence, as shown in Fig. 1A. Successful targeted integration was confirmed by Southern blot analysis as the appearance of a novel 4-kilobase pair SacI genomic fragment (Fig. 1B). To generate ATM−/−/c-Abl−/− mutant clones, two c-Abl disruption constructs, pAABL-his and pAABL-bsr, were sequentially transfected into ATM−/− mutant clones (20). The disruption of the ATM and c-Abl genes was finally verified by RT-PCR analysis (Fig. 1C).

c-Abl, which is activated by ATM (6), has been shown to phosphorylate Rad51 on tyrosine in response to DNA damage (18, 19). However, the biological significance of these findings in HR DNA repair is not yet clear, as c-Abl-mediated phosphorylation negatively affected Rad51 activity in one set of experiments (18) but enhanced its association with Rad52 in another experiment (19). To further study a possible role for c-Abl in HR DNA repair, we analyzed the appearance of Rad51−/− in ATM−/− DT40 cells, as well as c-Abl−/− and ATM−/−/c-Abl−/− DT40 cells, following γ-irradiation. Rad51−/− foci are subnuclear aggregates, believed to represent intermediate structures formed during the recombination required to repair radiation-induced or replication-associated DNA damage (23-25). Because ATM−/− DT40 cells displayed various double-stranded break (DSB) DNA repair defects, we first examined whether the loss of ATM function had any effect on this activity. We immuno-stained radiation-induced Rad51 foci at different times in wild-type and ATM-null DT40 cells and visualized them using confocal microscopy. As shown in Fig. 2, the disruption of ATM led to a pronounced delay in the formation of Rad51 foci, further indicative of dysfunctional HR in ATM-deficient cells. However, in c-Abl−/− DT40 cells Rad51 foci formed at a normal rate, and there was no significant difference in Rad51 focus formation between ATM−/− and ATM−/−/c-Abl−/− DT40 cells (Fig. 2). These results, although not in direct conflict with the demonstration that c-Abl inhibits binding of Rad51 to DNA (18), are in contrast to the report that c-Abl enhances the association of Rad51 with DNA (20).

To analyze the DSB DNA repair capacity of c-Abl−/− DT40 cells, the wild-type and mutant clones on methyleneblue plates were irradiated with various x-ray doses, and the percentage of clones surviving was determined relative to the numbers of colonies arising on untreated plates. As shown in Fig. 3A, ATM−/− DT40 cells were extremely x-ray-sensitive, as we have recently reported (20). However, c-Abl−/− DT40 cells did not exhibit hypersensitivity to x-ray, and c-Abl disruption did not enhance the x-ray sensitivity of ATM−/− DT40 cells (Fig. 3A).

To confirm the results of this x-ray sensitivity assay, we studied chromosomal aberrations in ATM−/− and c-Abl−/− DT40 cells following IR treatment. Because the IR DNA repair pathway is used to repair induced DNA damage in the S-G2 phase of the cell cycle (22), we reasoned that an increase in induced chromosomal aberrations observed within 3 h after IR (i.e. cells irradiated during the S-G2 phase of the cell cycle)
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would reflect a defect in the HR repair pathway. As shown in Fig. 3B, ATM<sup>−/−</sup> DT40 cells displayed highly increased levels of chromosomal aberrations within 3 h of x-ray irradiation, as we have recently reported (20). However, no significant increase in chromosomal aberration frequencies was observed in c-Abl<sup>−/−</sup> DT40 cells compared with wild-type cells. In addition, disruption of c-Abl in ATM<sup>−/−</sup> DT40 cells did not lead to an increase in the ATM-related defects in chromosomal maintenance (Fig. 3B).

Although the above described results indicate that c-Abl disruption did not grossly impair a cell’s DSB repair capacity, it was of interest to study the possible role of c-Abl in another important cellular response to IR, apoptosis (11, 12, 13). For quantitative analysis of IR-induced apoptosis, we monitored the initial phase of apoptosis, using annexin V staining at 4 h following irradiation and subsequent flow cytometric analysis. As shown in Fig. 4, the frequencies of spontaneous apoptosis were slightly but significantly increased in both ATM<sup>−/−</sup> and ATM<sup>−/−</sup>/c-Abl<sup>−/−</sup> DT40 cells compared with wild-type cells. This slight increase in spontaneous apoptosis in ATM-deficient DT40 cells is consistent with our recent findings that ATM-deficient DT40 cells show a slight p53-independent proliferation impairment (20), which presumably results from the elevated levels of spontaneous chromosomal aberrations in these cells (Fig. 3B) (20). Interestingly, whereas the amount of IR-induced apoptosis was slightly increased in ATM<sup>−/−</sup> DT40 cells, IR-induced apoptosis was inhibited in c-Abl<sup>−/−</sup> DT40 cells. Furthermore, ATM disruption in c-Abl<sup>−/−</sup> DT40 cells did not result in enhancement of the IR-induced apoptosis but rather in inhibition (Fig. 4). These results indicate important roles for c-Abl in IR-induced apoptosis, in agreement with the results of recent studies in human and murine cells (11–13).

Finally, we examined the possibility that the observed defect in IR-induced apoptosis reflects a role for c-Abl in cell cycle checkpoint control. Because DT40 cells do not express p53, wild-type DT40 cells accumulate in the G<sub>2</sub>-M phase but not in the G<sub>1</sub> phase following IR, and ATM disruption results in defective IR-induced mitotic delay (20). However, normal IR-induced mitotic delay was observed in c-Abl<sup>−/−</sup> DT40 cells, and c-Abl disruption did not exacerbate the IR-induced defective mitotic delay of ATM<sup>−/−</sup> DT40 cells (data not shown). These results therefore preclude the possible involvement of G<sub>2</sub>-M checkpoint control abnormalities in the defective apoptotic response in c-Abl-deficient DT40 cells.

In the present study, we have shown that the accumulation of Rad51 foci was slower in ATM-deficient cells than in wild-type cells, although the number of foci increased with time and gradually exceeded those found in similarly treated wild-type cells. This latter increase in Rad51 foci has been noted in A-T cells (26) and may reflect the accumulation of un- or improperly repaired DNA lesions. Strikingly, the formation of foci of other DSB repair proteins, the Mre11-Rad50 complex, in response to irradiation of A-T cells, is severely compromised (26), further indicating a possible defect in DNA repair capacity caused by ATM defects (27, 28) in addition to the cell cycle abnormalities characteristic of A-T. However, although c-Abl, activated directly by ATM (6), has been shown to modulate Rad51 activity in response to DNA damage (18, 19), Rad51 focus formation was normal in c-Abl-deficient cells. In support of these results, DSB repair capacity in c-Abl-deficient cells was not grossly impaired as assessed by IR sensitivity and IR-induced chromosomal breakage analyses, although various DSB repair defects have been well documented in ATM-deficient cells (20). These results indicate therefore that, although ATM (20) and Rad51
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(17) are indispensable in DSB repair in eukaryotic cells, there must be redundant functions for c-Abl in DSB repair, at least in chicken DT40 cells and mouse fibroblasts (29). Alternatively, it is possible that the c-Abl-related gene (30) substitutes for c-Abl in the c-Abl−/− cells in genome maintenance or G2-M checkpoint control. In contrast to its redundant role in DSB repair, c-Abl appears to play an important role in IR-induced apoptosis. However, as DT40 cells do not express p53 (20), it is likely that the c-Abl targets in IR-induced apoptosis are p53-related effectors such as the recently described p73 (12, 13). Further work is required to define the precise functions of c-Abl in DNA damage-induced apoptosis.

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