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ATR–ATRIP Kinase Complex Triggers Activation of the Fanconi Anemia DNA Repair Pathway

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Abstract

ATR kinase activates the S-phase checkpoint when replication forks stall at sites of DNA damage. This event also causes phosphorylation of the Fanconi anemia (FA) protein FANCI, triggering its monoubiquitination of the key DNA repair factor FANCD2 by the FA core E3 ligase complex, thereby promoting this central pathway of DNA repair which permits replication to be restarted. However, the interplay between ATR and the FA pathway has been unclear. In this study, we present evidence that their action is directly linked, gaining insights into this relationship in a DT40 mutant cell line that is conditionally deficient in the critical ATR-binding partner protein ATRIP. Using this system, we showed that ATRIP was crucial for DNA damage-induced FANCD2 monoubiquitination and FANCI phosphorylation. ATR kinase phosphorylated recombinant FANCI protein in vitro, which was facilitated by the presence of FANCD2. Mechanistic investigations revealed that the RPA region but not the TopBP1 region of ATRIP was required for FANCD2 monoubiquitination, whereas Chk1 phosphorylation relied upon both domains. Together, our findings identify ATR as the kinase responsible for activating the FA pathway of DNA repair. Cancer Res; 72(5); 1149–56. ©2012 AACR.

Introduction

Fanconi anemia (FA) is a genome instability disorder characterized by increased incidence of cancer, progressive bone marrow failure, developmental abnormalities, and hypersensitivity to DNA interstrand cross-links (ICL; refs. 1–3). FA is caused by a mutational defect in the common FA pathway, which is composed of at least 15 FA proteins (3). The central component of the pathway is the FA core complex, which contains at least 8 FA (FANCA/B/C/E/F/G/L/M) proteins and 4 FA-related factors (FAAP24, FAAP100, MHLF1, and MHLF2; refs. 3–5). In response to replication stress and during S phase, the FA core complex functions as an E3 ligase to monoubiquitinate the FANCD2/FANCI (ID) complex, which is then loaded onto damaged chromatin to facilitate DNA repair (6, 7). In ICL repair, FANCD2 has a role in making incisions at both sides of the damage and promoting translesion DNA synthesis across the incised ICL (8, 9). Recent studies showed that monoubiquitinated FANCD2 recruits critical effector molecules such as the Fan1 nuclease or SLX4 via their ubiquitin-binding domains (2, 3, 10). Thus, monoubiquitination of FANCD2 is critical not only for its chromatin localization but also for acting as a scaffold for specific DNA repair factors.

Our previous study indicated that DNA damage–induced FANCI phosphorylation at the S/TQ cluster domain serves as a molecular switch to activate FANCD2 monoubiquitination (11). Three S/TQ-directed phosphoinositide 3-kinase (PI3K)-related kinase family members ATM, DNA-PK, and ATR play partially overlapping but distinct roles in the DNA damage response (12). ATR is activated by single-stranded (ss) DNA exposed during replication stress, whereas ATM and DNA-PK respond primarily to DNA double strand breaks. A critical interacting partner of ATR, ATRIP protein, mediates the accumulation of ATR on damaged chromatin via an interaction with the RPA complex, which recognizes and coats ssDNA (13). In parallel, the Rad9-Rad1-Hus1 (9-1-1) checkpoint clamp, loaded onto DNA by the RAD17-replication factor C clamp loader complex, recruits TopBP1 (14). These serial actions result in the catalytic activation of ATR through direct binding of ATR–ATRIP with the activation domain of TopBP1. The ATR–TopBP1 interaction is further stabilized by ATR autophosphorylation on S1989 (15). Active ATR results in...
phosphorylation and activation of Chk1, which in turn further orchestrates the biological responses to DNA damage.

It is of interest to determine which kinase is actually responsible for the phosphorylation of FANCI. Although it seems reasonable to assume that the ATR kinase plays an important role in the FA pathway, the issue has not been settled. Here, we clarify the role of the ATR–ATRIP kinase in the FA pathway with a genetically defined system.

Materials and Methods
Gene targeting in DT40 cells
Culture and transfections in DT40Cre1 were carried out as described (11). DT40Cre1, a subline of DT40-expressing MerCreMer (16), was obtained from Drs. Jean-Marie Buerstedde and Hiroshi Arakawa and has been maintained by M. Takata. This cell line has been repeatedly verified in our laboratory by several criterion including morphology, karyotype, drug sensitivity, and gene targeting efficiency. To make conditional ATRIPflOX-GFP/- cells (Fig. 1A and Supplementary Fig. S1), one of the ATRIP alleles was disrupted by gene targeting by replacing exons encoding chicken ATRIP amino acids 364 to 376 with the Blastocidin resistance gene (bsr) cassette, which was then removed by TAM-activated Cre. Afterward, a pair of the loxP sequence was knocked into the other allele so that the exons encoding the C-terminal ATR-binding domain (amino acids 614 to 822) could be floxed. The GFP sequence was also knocked-in at the termination codon to monitor the expression of endogenous ATRIP protein. Removal of the bsr cassette by transient expression of the Flp recombinase (Flp expression plasmid was provided by Dr. Kyoji Horie, Osaka University) resulted in establishment of ATRIPflOX-GFP/- cells. The cells were characterized after 20 nmol/L TAM treatment for 48 hours unless otherwise stated.

Expression vectors
Full-length chicken ATRIP cDNA was amplified by reverse transcriptase PCR from DT40 RNA and cloned into pDONR vector (Invitrogen). The sequence data were deposited at the DDBJ database (accession number AB684452). Mutations were introduced by the QuickChange Kit (Stratagene). After sequencing, the gateway system (Invitrogen) was used to transfer the CDNA to the GFP expression vector containing a nuclear localization signal (NLS). GFP-chicken FANCI expression vector has been described elsewhere (11).

Antibodies and reagents
Anti-chicken FANCD2 (17) and anti-Rad51 (18) serum have been described. Anti-chicken FANCI serum was raised by immunizing rabbits with GST fusion protein containing chicken FANCI (1-251 amino acid region). Other antibodies were purchased from Clontech (polyclonal anti-GFP), Santa Cruz Biotechnology (polyclonal anti-ATR, monoclonal anti-Chk1), Sigma (monoclonal anti-FLAG M2 antibody), or Cell Signaling Technology (monoclonal anti-phospho-Chk1-Ser345). ATM inhibitor Ku55933 and Chk1 inhibitor UCN-01 was purchased from CalbioChem and Sigma, respectively.

Western blotting
Cells were treated with the indicated dose of TAM and/or MMC (Kyowa-hakkou-Kirin) and lysed in SDS sample buffer. Samples were separated by polyacrylamide gel electrophoresis, transferred to a membrane, and detected with indicated antibodies and ECL reagents (GE Healthcare). Phostag-Western blotting was carried out as described (11).

Analysis of growth and cell sensitivity toward cisplatin
Cell cycle and cell proliferation rate with plastic microbeads was assessed as described (19). Cell viability in liquid culture containing cisplatin (Nippon Kayaku) was assessed after 48 hours using FACS Calibur (BD) and propidium iodide (PI) staining (11).

Subnuclear focus formation assay
After MMC exposure, cytospin slides were fixed with 4% paraformaldehyde/PBS and stained with antibodies against chicken FANCD2 or FANCI followed by Alexa Fluor 594-conjugated secondary antibody (Invitrogen) with DAPI counterstaining. Images were captured by fluorescent microscopy (DM2500B; Leica) or confocal laser scanning microscopy (TCS SP5; Leica).
Purification of recombinant chicken FANCD2 and FANCI

Briefly, the DNA fragment encoding chicken FANCD2, wild-type FANCI, or phospho-mimic mutant FANCI (Dx6) was ligated into the pET-15b vector, and was overexpressed in the Escherichia coli BL21(DE3) strain (Codon(+)RIL; Stratagene). The cells were resuspended in buffer A containing 50 mmol/L Tris-HCl (pH 8.0), 10% glycerol, 0.5 mol/L NaCl, 1 mmol/L PMSF, 12 mmol/L imidazole, and 5 mmol/L 2-mercaptoethanol, and disrupted by sonication. His6-tagged FANCD2 or FANCI was purified with nickel-nitrilotriacetic acid agarose resin, a Heparin Sepharose CL-6B column (GE Healthcare), and Superdex 200 gel filtration column (GE Healthcare). The His6 tag was removed by digestion with thrombin protease (GE Healthcare). The ID complex was prepared by mixing FANCD2 and FANCI preparations at 1:1 stoichiometry.

In vitro kinase reaction

Cloning and expression of chicken ATR that has been N-terminally HFSC tagged in DT40 cells lacking endogenous ATR will be described elsewhere (JE, FP, and NFL; unpublished results). Cells were lysed in lysis buffer (0.5% Triton X100, 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 25 mmol/L NaF, 25 mmol/L β-glycerophosphate, 10% glycerol, 1 mmol/L Na3VO4, 1 mmol/L DTT, 1 mmol/L PMSF) containing proteinase inhibitor tablet (Roche), and HFSC-ATR was immunoprecipitated using anti-FLAG-beads (Sigma). Following washes, the immunoprecipitates were incubated in kinase buffer [10 mmol/L HEPES, pH 7.4, 50 mmol/L β-glycerophosphate, 50 mmol/L NaCl, 10% glycerol, 10 mmol/L MgCl2, 10 mmol/L MnCl2, 1 mmol/L DTT, 6 mmol/L Na3VO4, 1 mmol/L PMSF, 5 mmol/L ATP, γ-32P-ATP (10 μCi per sample)] containing proteinase inhibitor tablet for 30 minutes at 30°C. Recombinant protein (1 μg) of chicken FANCD2, FANCI, FANCD2/FANCI complex, or GST-p53 (kindly provided by Dr Junya Kobayashi) purified from E. coli was added as exogenous substrate. Samples were separated using 5% to 20% gradient SDS-PAGE gel.

Results and Discussion

To test the function of ATR-ATRIP, we have constructed a conditional ATRIP knockout cell line ATRIPflox-GFP/C0 using DT40Cre1 (Fig. 1A and Supplementary Fig. S1), which express the tamoxifen (TAM)-inducible Cre recombinase called MerCreMer (16). In this cell line, the exons encoding the ATR-binding domain of ATRIP (Supplementary Fig. S2) are deleted upon addition of TAM. This process can be monitored visually as a GFP tag was also knocked-in at the termination codon of ATRIP (Fig. 1A). Previous studies showed that the MerCreMer-loxP system works efficiently in shutting off a gene of interest (20, 21). Indeed, as shown in Fig. 1B, the expression of ATRIP-GFP was almost abrogated at the 24 hour time point following TAM addition, and completely undetectable after 48 hours. The growth of the cells was abrogated (Fig. 2A) and a
significant amount of dead cells (with sub-G1 DNA content) appeared (Fig. 2B) at 72 hours. TAM treatment also resulted in fewer MMC-induced ATRIP-GFP foci (Fig. 2C). For all further experiments, we decided to use cells treated with TAM for 24 or 48 hours to examine activation of the FA pathway.

We first confirmed both wild-type DT40Cre1 and untreated ATRIP<sup>flox-GFP</sup>– cells displayed similar levels of monoubiquitination of the ID complex induced by MMC stimulation (Fig. 3A). Robust Chk1 phosphorylation also occurs as detected by anti–phospho-Ser345-Chk1 antibody (Fig. 3A). When ATRIP<sup>flox-GFP</sup>– cells were treated with TAM for 48 hours followed by an additional treatment with MMC for 6 hours, Chk1 phosphorylation was undetectable consistent with loss of ATRIP expression (Fig. 3A). Most importantly, the monoubiquitination of FANCD2 and FANCI induced by MMC was dramatically reduced (Fig. 3A). We also examined FANCI phosphorylation using Phostag Western blotting as we have done in our previous report (11) and found that it was undetectable in ATRIP-depleted cells (Fig. 3B).

One possible explanation for this reduced monoubiquitination was that there are less G2 cells in MMC-treated ATRIP-depleted cells due to impaired G2 arrest. As shown in Fig. 3C and Supplementary Fig. S3, we indeed found a difference between ATRIP-proficient and ATRIP-deficient cells staying at G2 phase following MMC treatment (65% vs. 32%). To examine whether the FANCID2 L/S ratio is affected by the decrease in G2 phase fraction, we treated ATRIP-proficient cells with Chk1 inhibitor UCN-01 to abrogate MMC-induced G2 arrest. Simultaneous treatment of ATRIP-proficient cells with MMC and UCN-01 resulted in decreased G2 phase fraction as expected (34%), the level similar to that of MMC-treated ATRIP-deficient cells (32%). However, the L/S ratio was only mildly affected by UCN-01 (1.82 vs. 1.72), and this was much higher than the reduced L/S ratio (0.33) in ATRIP-deficient cells (Fig. 3C). Therefore, we conclude that the reduction in the FA pathway activation in ATRIP-deficient cells is not accounted for by the defective G2 arrest.

Figure 3. Analysis of the DNA damage response in ATRIP<sup>flox-GFP</sup>– cells. A, monoubiquitination of FANCID2 or FANCI and Chk1 phosphorylation. Cells were treated with TAM and then stimulated with MMC (100 ng/mL for 6 hours) and analyzed by Western blotting. The monoubiquitinated (L) or nonubiquitinated (S) form is indicated. B, MMC-induced FANCI phosphorylation as analyzed by Phostag Western blotting. Cells were treated with or without TAM (200 nmol/L for 24 hours) and then stimulated with MMC (100 mg/mL for 6 hours). Partially degraded FANCI was observed (indicated with a white arrowhead) in TAM-treated cells. C, the FANCD2 L/S ratio and G2 cell fraction. TAM-treated or nontreated ATRIP<sup>flox-GFP</sup>– cells were stimulated with MMC 100 ng/mL and/or UCN-01 300 nmol/L for 6 hours and the cell-cycle profile and the L/S ratio were examined. D, foci formation of ATRIP-GFP, FANCD2, and FANCI. Following TAM treatment and MMC damage (100 ng/mL for 6 hours), cells were fixed and observed. The bar graph represents %FANCID2– or FANCI– positive cells. One hundred nuclei were scored, and nuclei containing more than 4 bright foci were defined as foci positive.
We noticed that small amounts of the L-form of the ID complex could still be observed after TAM treatment (Fig. 3A and B). It was possible that the remaining I-D2 monoubiquitination could be due to ATM, which may substitute for ATR in MMC-induced activation of the FA pathway. However, specific pharmacologic inhibition (Ku55933) of ATM did not further downregulate the levels of FANCD2 monoubiquitination in TAM-treated ATRIP<sup>lox-GFP</sup>/− cells (Supplementary Fig. S4). We speculated that deubiquitination of the ubiquitinated ID complex by USP1 may be inefficient and a recent structural study supports this might be the case, as the lysine-ubiquitin isopeptide bond is protected from USP1 by the sequestration at the ID interface (22). Alternatively, the efficient deubiquitination by USP1 may require ATR–ATRIP kinase because the phosphorylation-regulated interaction between USP1/UAF1 complex and FANCI is suggested to be important for the deubiquitination (23).

We further observed that MMC-induced ATRIP foci colocalized very well with FANCD2 or FANCI foci, and loss of ATRIP resulted in abrogation of the ID foci formation (Fig. 3D). We also observed that stable expression of a FANCI phosphomimetic mutant (FANCI Dx6) in which 6 potential phosphorylation sites in the S/TQ cluster domain have been mutated to aspartic acid (11) partially rescued the loss of FANCD2 mono-ubiquitination caused by ATRIP deletion (Supplementary Fig. S5). This observation further supports our reasoning that FANCI phosphorylation is required to trigger FANCD2 monoubiquitination.

We next examined whether ATR can directly phosphorylate FANCI protein in vitro. We used chicken ATR immunoprecipitated with an anti-FLAG antibody from DT40 cells stably expressing chicken ATR amino terminally tagged with multiple epitopes (HA, FLAG, Strep TagII and Calmodulin-binding protein, termed HFSC-ATR) and recombinant chicken FANCI, FANCD2, or the FANCI–FANCD2 complex. Of note, the 2-step (FLAG-Strep tag) purified ATR preparation contained significant amount of ATRIP as shown by a silver-stained gel (Supplementary Fig. S6) and mass spectrometric analysis (J.E. and N.L., unpublished results), similarly to the human ATR–ATRIP complex (24). We were able to detect autophosphorylation of ATR and the phosphorylated protein species corresponding to the phospho-ATRIP, phospho-FANCD2, and phospho-FANCI in these in vitro kinase assays (Fig. 4A). As expected, the mutant FANCI protein (Dx6 protein; ref. 11) could not be phosphorylated (Fig. 4B). Phosphorylation of FANCD2 and FANCI was much less efficient compared with GST-p53 (data not shown). However, the phosphorylation on wild-type FANCI, but not on the Dx6-mutant FANCI, was significantly enhanced by the addition of FANCD2 (Fig. 4A and B). This observation is consistent with our recent findings that efficient in vivo phosphorylation of FANCI requires FANCD2 as well as the intact FA core complex (Tomida and colleagues, submitted). It would be interesting to test whether the addition of the purified FA core complex could enhance the efficiency of the in vitro kinase reaction.

To further elucidate how ATRIP participates in activation of the FA pathway, we expressed wild-type or mutant chicken ATRIP (Fig. 5A) in ATRIP<sup>lox-GFP</sup>/− cells. The N-terminal deletion mutant lacking amino acids 1-108 (hereafter designated ATRIP-rpa) is expected to abrogate RPA binding because this region contains the conserved acidic amino acids (see Supplementary Fig. S2) known to interact with RPA70 (25–27). The LLSS45AAAA mutation (designated ATRIP-top) should disrupt interaction with TopBP1, as these 4 residues (LLSS) are completely conserved between human and chicken ATRIP (Supplementary Fig. S2) and have been reported to be required for the ATRIP–TopBP1 interaction in human cells (28). Note that the ATRIP expression constructs included an NLS and GFP at their C-terminus, and transfectants were selected by flow cytometry on the basis of increased GFP fluorescence.

Upon switching off expression of integrated ATRIP-GFP by treatment of cells with TAM, the wild-type ATRIP transgene could complement the cells with respect to both Chk1 phosphorylation and FANCD2 monoubiquitination following treatment with MMC (Fig. 5B). In contrast, Chk1 phosphorylation could not be induced in cells expressing ATRIP-rpa or ATRIP-top transgenes (Fig. 5B). Interestingly, robust FANCD2 monoubiquitination was not supported by the ATRIP-rpa transgene, whereas cells expressing ATRIP-top were fully proficient for this modification (Fig. 5B). The latter finding is consistent with our recent observation that chicken cells lacking RAD17 or human cells treated with siRNA targeting TOPBP1 were still able to induce FANCD2 monoubiquitination (Tomida and colleagues, submitted). Furthermore, colocalization of ATRIP–FANCD2 foci was abrogated in cells expressing ATRIP-rpa, but not the ATRIP-top mutant (Fig. 5C). These results indicated that the association between ATRIP and TopBP1 is dispensable for FANCD2 monoubiquitination and hence for

Figure 4. FANCI phosphorylation in in vitro ATR kinase reaction. Chicken HFSC-tagged ATR were immunoprecipitated with anti-FLAG and subjected to in vitro kinase reaction. Anti-FLAG immunoprecipitates from wild-type DT40 cells was used as negative control. Recombinant chicken FANCI and FANCD2 (A) or either wild-type FANCI(WT) or phospho-mimic FANCI (Dx6) and FANCD2 (B; 1 μg per reaction) were added as indicated. An asterisk indicates a nonspecific phosphorylation band. CBB, Coomassie brilliant blue staining.
FANCI phosphorylation as well. Conversely, the interaction between ATRIP and RPA is essential for the function of the ATR–ATRIP kinase complex. Indeed, we observed that ATRIP-rpa mutant as the sole source of ATRIP could not sustain cell proliferation, indicating that the ATRIP–RPA interaction is required for the function of the ATR kinase in cycling cells that have not been treated with any exogenous agents (Supplementary Fig. S7).

We also tested cisplatin sensitivity of ATRIP \textsuperscript{fl}ox-GFP/\textsuperscript{−} cells and their derivatives expressing ATRIP mutants (Fig. 6). Cells treated for 24 hours with TAM were exposed to cisplatin for an additional 48 hours and then cell viability was determined with flow cytometry. ATRIP\textsuperscript{fl}ox-GFP/\textsuperscript{−} cells rendered deficient for ATRIP by treatment with TAM were sensitive to cisplatin compared with wild-type cells or untreated ATRIP\textsuperscript{fl}ox-GFP/\textsuperscript{−}. After TAM-induced removal of endogenous ATRIP, cells expressing either ATRIP-rpa or ATRIP-top, but not wild-type ATRIP, were also sensitive to cisplatin similar to results obtained with cells defective in \textit{FANCC} (29), which encodes a component of the FA core complex.

There has been controversy in the literature about the role of ATR in the activation of the FA pathway (30–34). Although at the moment it is impossible to exclude an unknown kinase other than ATR or ATM playing a small role, our data collectively indicate that ATR–ATRIP kinase is critical in triggering the FA pathway activation via FANCI phosphorylation. FANCI phosphorylation is likely mediated by a direct action of ATR because ATR can phosphorylate FANCI \textit{in vitro}. The \textit{in vitro} dependence of FANCI phosphorylation on FANCD2 observed in this study supports our data as we have observed a similar dependency \textit{in vivo} (Tomida and colleagues, submitted). The precise mechanisms of ATR activation still remain elusive, however, our observation that the mechanism of FANCI phosphorylation by ATR is independent of TopBP1 suggests that it
should be distinct from that of Chk1. This also suggests that ATR activation can occur without TopBP1 association, in contrast to the widely accepted model (13). Supporting this notion, a recent study showed that ATR autophosphorylates itself in a TopBP1-independent manner (15). Additional studies on ATR signaling will be required to provide further novel insights into the activation of the FA pathway as well as checkpoint regulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Figure 6. Cisplatin sensitivity caused by loss or mutation of ATRIP in DT40 cells. Indicated cells were cultured for 48 hours in the medium containing cisplatin. Cell viability was assessed with flow cytometry and normalized to cells not treated with cisplatin.
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