FANCD2 is a target for caspase 3 during DNA damage-induced apoptosis

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The Fanconi anemia (FA) pathway, of which the FANCD2 protein is a key component, plays crucial roles in the maintenance of hematopoietic stem cells and suppression of carcinogenesis. However, the function of FANCD2 remains unclear. Here, we report that FANCD2 is a novel and specific substrate of caspase 3. Cleavage of FANCD2 by caspase 3 did not require either the FA core complex or mono-ubiquitylation of FANCD2, and was stimulated by p53. In addition, we identified the cleavage sites and generated cell lines that stably express a caspase-resistant FANCD2 mutant. Our data suggest that FANCD2 is regulated by caspase-mediated degradation during apoptosis induced by DNA damage.

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1. Introduction

Fanconi anemia (FA) is a rare genetic disorder associated with bone marrow failure, multiple skeletal malformations, and cancer susceptibility [1]. At the cellular level, FA is characterized by genome instability and hypersensitivity to DNA interstrand crosslinks (ICL) induced by chemotherapeutic platinum compounds (e.g., cisplatin) and bifunctional alkylating agents, such as mitomycin C (MMC). To date, 16 FA genes have been identified, and all of the encoded proteins function in an intracellular signaling pathway, the FA pathway, which regulates responses to and repair of ICL damage [2,3]. Recent studies have suggested that the FA pathway counteracts DNA damage induced by endogenous aldehydes in hematopoietic stem cells [4,5]. Eight of the FA gene products and other non-FA proteins associate with each other, forming the FA core complex [2,3]. Upon ICL damage, the FA core complex is loaded onto damaged chromatin [6], where it functions as a multi-subunit E3 ubiquitin ligase to mono-ubiquitylate both FANCD2 and FANCI proteins, which form a heterodimeric complex (ID complex) [2,3]. This mono-ubiquitylation step is a critical event in the FA pathway [7,8], because the mono-ubiquitylated ID complex is loaded at damaged sites by binding to aberrant DNA structures, where it forms subnuclear foci and orchestrates DNA repair [2,3,9]. The FA pathway is inactivated in a wide variety of human cancers through hyper-methylation of the promoter sequence of one of the FA genes, FANCF [10–13]. This inactivation causes cisplatin sensitivity, suggesting that the FA pathway plays a broad and important role in genome maintenance and prevention of carcinogenesis. Furthermore, reactivation of the FA pathway can contribute to acquired resistance to cisplatin in cancer cells [14,15]. These findings indicate that the integrity of the FA pathway is an important determinant of cellular responses to chemotherapy based on ICL-inducing compounds, which are widely used as anti-cancer drugs. The mechanism of action of those chemotherapeutic agents involves covalent binding to purine DNA bases, leading primarily to programmed cell death (apoptosis) [16]. During apoptosis, the caspase family of cysteine proteases is activated, and these factors mediate endolytic cleavage of many protein substrates. Regardless of what triggers the signal, apoptotic cells develop a characteristic morphology, including DNA fragmentation and loss of mitochondrial membrane potential. These changes are largely mediated by cleavage of caspase substrates. A large number of caspase substrates have been identified so far [17], and the list continues to grow. Many DNA damage response (DDR) proteins, such as BRCA1, CHK1, MDC1, MLH1, and RAD51, are caspase substrates [18–23], raising the possibility that cleavage of DDR proteins is critical for execution of apoptosis.
Here, we report that FANCD2, a key component of the FA pathway, is a novel and specific substrate of caspase 3 that is activated during apoptosis induced by DNA damage. This cleavage does not depend on either the functional FA core complex or mono-ubiquitylation of FANCD2, and it is stimulated by the tumor suppressor p53. Furthermore, we show that a mutant FANCD2 that is resistant to caspase cleavage exerts normal functions in DNA repair, except for formation of damage-induced nuclear foci, and does so without significantly affecting apoptosis.

2. Materials and methods

2.1. Cell cultures and chemicals

The FA-deficient cell lines were grown in DMEM supplemented with 15% FBS. The other cells were all grown in DMEM supplemented with 10% FBS. Cisplatin (P4394), z-VAD-fmk (C2105), and methyl methanesulfonate (MMS; 125, 250, and 500 mM) were purchased from Sigma–Aldrich. Lactacystin (426102) and staurosporine (569396) were purchased from Calbiochem. Mitomycin C was purchased from Nacalai Tesque (20898-21).

2.2. Immunoblotting, immunoprecipitation and immunofluorescence

Immunoblotting, immunoprecipitation and immunofluorescence were performed as described in the Supplemental data.

2.3. Retroviral infection

Production of pMMP retroviral supernatants and infection of cells were performed as previously described [24].

2.4. Site-directed mutagenesis

Site-directed mutations were introduced with the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). The mutations were verified using the BigDye Terminator v3.1 Cycle Sequencing Kit and the 3130 Genetic Analyzer (Life Technologies).
2.5. Cell-free caspase assay

Cell-free caspase assays were carried out with solutions and enzymes, which were all purchased from BioVision. Whole-cell extract was prepared using Cell Lysis Buffer (1067-100). Aliquots of the lysate were incubated for 1 h at 37 °C with active forms of individual recombinant caspases (K233-10-25) in 1× Reaction Buffer (1068-20).

2.6. shRNA expression

All shRNAs were expressed using the pSIREN-RetroQ system (Takara Bio). The individual shRNA constructs were designed using the RNA Target Sequence Selector (Takara Bio). Each sequence is shown in Supplemental data.

2.7. Mitomycin C sensitivity assay

Cells were seeded onto 10 cm dishes (500 cells/dish). After overnight culture, cells were treated with the indicated concentrations of MMC for 24 h. After washing with phosphate-buffered saline (PBS), cells were cultured in normal medium for 10–14 days. The resultant colonies were visualized by fixation and staining with 1% crystal violet. Only colonies with more than 50 cells were scored.

3. Results

3.1. FANCD2 is cleaved by caspases during apoptosis

The FANCD2 protein is mono-ubiquitylated upon DNA damage [7]. Consistent with this notion, in HeLa cells cultured under normal conditions, immunoblot analyses mainly revealed non-ubiquitylated FANCD2 (FANCD2 short form: D2-S), whereas mono-ubiquitylated FANCD2 (FANCD2 long form: D2-L) was induced by treatment with various genotoxic stresses (Fig. 1A). However, particularly with relatively high doses of DNA damage, we observed attenuation of both forms of FANCD2 and simultaneous appearance of new bands with higher mobility that reacted with anti-FANCD2 antibody (D2-XS). These bands were observed irrespective of the DNA-damaging agent employed and at relatively low doses of these agents (Fig. 1A). However, high doses of DNA-damaging agents were used to facilitate the detection of cleavages. To confirm that these smaller polypeptides were indeed derived from FANCD2, we stably expressed EGFP-tagged FANCD2 in the FANCD2-deficient cell line, PD20F. After treatment with a relatively high dose of ultraviolet light (UV), we observed a similar pattern of high-mobility bands that reacted with anti-GFP antibody (Fig. 1B, lane 6), indicating DNA damage-induced cleavage of FANCD2. A pan-caspase inhibitor, z-VAD-fmk, suppressed the appearance of the shorter species of FANCD2 (Fig. 1C, lanes 5 and 6), whereas a proteasome inhibitor, lactacystin, slightly increased the levels of this species (Fig. 1C, lanes 3 and 4, and Fig. S1). Notably, simultaneous treatment with z-VAD-fmk and lactacystin suppressed UV-induced cleavage of FANCD2 (Fig. 1C, lanes 7 and 8) as well as treatment with z-VAD-fmk alone. When the shorter forms of FANCD2 were detectable, the active forms of caspase 3 and 7 were also observed (Fig. 1C), indicating that extensive DNA damage had induced apoptosis in these cells. Treatment with the pan-kinase inhibitor staurosporine (known to be an apoptosis inducer) also induced the same pattern of shorter forms of FANCD2 (Fig. 1C, lane 9). These results indicate that the FANCD2 protein is cleaved by caspases during apoptosis upon relatively high levels of DNA damage.

3.2. FANCD2 is cleaved specifically by caspase 3

To identify the caspase responsible for the FANCD2 cleavage, we carried out cell-free caspase assays using active forms of recombinant caspases. Whole-cell extracts from normal human fibroblasts (without treatment with DNA-damaging agents) were incubated individually with each of recombinant caspases. Immunoblot analyses of endogenous FANCD2 (Fig. 2) revealed that caspases 1, 3, 6, 7, and 8 could cleave FANCD2 (Fig. 2). Those cleavages were completely suppressed in the presence of z-VAD-fmk. In particular, the cleavage pattern by caspase 3 seemed almost the same as that observed in vivo. To confirm this notion in vivo, we depleted caspase 3 in HeLa cells using shRNA. As shown in Fig. 3A, depletion...
of caspase 3 suppressed FANCD2 cleavage even after high-dose UV (lanes 5 and 6), whereas depletion of caspase 7 had only a marginal effect (Fig. 3B, lanes 5 and 6). Furthermore, the breast cancer cell line MCF-7, which lacks caspase 3 [25,26], did not exhibit detectable FANCD2 cleavage after treatment with any DNA-damaging agent, despite the presence of the active form of caspase 7 (Fig. 3C). Together, these results indicate that FANCD2 is cleaved specifically by caspase 3.

3.3. Effect of the FA core complex and p53 on FANCD2 cleavage

In response to DNA damage, mono-ubiquitylation of FANCD2 is mediated by the FA core complex. This post-translational modification of FANCD2 is important for downstream events in the FA pathway [7]. To determine whether the FA core complex is required for FANCD2 cleavage, we took advantage of cell lines lacking functional FANCA, FANCC, or FANCF, all of which are essential components of the FA core complex. When these cells were treated with a relatively high dose of cisplatin, FANCD2 cleavage was observed in all cell lines (Fig. 4A). Although the mono-ubiquitylation of FANCD2 could be restored in these cells by expression of the corresponding wild-type FA genes, FANCD2 cleavage was not affected by this functional complementation. Furthermore, mutation at the mono-ubiquitylation site in FANCD2 (K561R) had no effect on cleavage (Fig. 4B). These results indicate that the FA core complex and mono-ubiquitylation of FANCD2 are dispensable for FANCD2 cleavage.

Upon DNA damage and other types of cellular stresses, the tumor-suppressor protein p53 regulates key processes, including DNA repair, cell-cycle arrest, and apoptosis [27]. To investigate the roles of p53 in FANCD2 cleavage, we analyzed a pair of HCT116 cell lines with either wild-type or disrupted p53 genes [28]. In the cell line with intact p53, DNA damage induced stabilization and robust accumulation of p53 (Fig. 5). Concomitant with accumulation of p53, we detected a reduction in the level of full-length FANCD2 (both L and S forms) and the appearance of its cleavage products (XS form). On the other hand, the cognate p53-deficient cell line was less able to induce FANCD2 cleavage, even in the presence of extensive DNA damage (Fig. 5, lanes 5–8 and lanes 13–16). These results indicate the possibility that functional p53 promotes DNA damage–induced apoptosis and the FANCD2 cleavage.

3.4. Mapping of FANCD2 cleavage sites

Caspases share specificity for the amino-acid sequences at cleavage sites of target proteins, typically cleaving at the carboxyl-terminal side of the last aspartic acid in the tetra-peptide sequences x-x-x-D or D-x-x-D [26]. Based on the pattern of the cleavage products (D2-XS), we hypothesized that FANCD2 possesses at least four target sites for caspase 3. To determine the cleavage sites, we performed site-directed mutagenesis to introduce D-to-A mutations at several predicted candidate sites in the FANCD2 sequence. These mutant FANCD2 genes were tagged with EGFP, and the resultant fusion protein was stably expressed in the FANCD2-deficient PD20F cell line. As shown in Fig. 6, a change in any one of four aspartic acids (D667, 693, 753, and 1046) abolished one of the major cleavage products of FANCD2, whereas the D662A mutation had no effect. Next, we simultaneously introduced all four relevant D-to-A mutations in the FLAG-tagged FANCD2 sequence, which was stably expressed in the FANCD2-deficient PD20F cell line. We confirmed that the quadruple (4DA) mutations in FANCD2 conferred almost complete resistance to DNA damage-induced cleavage (Fig. 7A).
Fig. 4. FANCD2 cleavage is independent of the FA core complex and its mono-ubiquitylation activity. (A) Three FA-deficient cell lines, GM6914 (FA-A), PD426F (FA-C), and TOV-21G (FANCF-inactivated ovarian cancer), as well as the corresponding complemented cell lines, were treated with various concentrations of cisplatin for 24 h. (B) Either wild-type FANCD2 (+wtD2) or K561R (mono-ubiquitylation site) mutant FANCD2 was stably expressed in PD20F cells, which were treated with the indicated concentrations of cisplatin for 24 h. Whole-cell extracts were subjected to immunoblotting with the indicated antibodies.

Fig. 5. p53 stimulates FANCD2 cleavage. A p53-proficient HCT116 cell line (WT) and a derivative line in which p53 was knocked out (KO) were cultured for 24 h after irradiation with the indicated doses of UV, or cultured for 24 h with continuous exposure to the indicated concentrations of cisplatin. Whole-cell extracts were subjected to immunoblotting with the indicated antibodies.
3.5. Functions of the caspase-resistant FANCD2

To examine the effect of cleaved FANCD2, a cell line expressing a C-terminal truncated form of FANCD2 was generated. However, expression of the truncated form had no effect on the cell survival of the parental PD20F cell line (Fig. S2). Furthermore, to assess the function of FANCD2 cleavage, we compared the DDR of cell lines stably expressing caspase-resistant FANCD2 with that of a control cell line expressing wild-type FANCD2. Both cell lines exhibited FANCD2 mono-ubiquitylation in response to cisplatin treatment (Fig. 7A), resistance to mitomycin C (MMC) (Fig. 7B), and interaction between FANCD2 and FANCI (Fig. 7C). In addition, as shown in Fig. 7D, the two cell lines expressing wild-type or mutant FANCD2 exhibited similar apoptotic responses to cisplatin treatment, judging from cleavage of PARP1 and caspase 3. These results indicate that the absence of FANCD2 cleavage did not affect ICL damage repair or apoptosis induction in these cells. Surprisingly, however, we noticed that the DNA damage-induced formation of nuclear foci was compromised in cells expressing the caspase-resistant mutant FANCD2, which was mostly diffusely distributed in the nucleus irrespective of the presence or absence of DNA damage (Fig. 7E and S3). These results not only indicate the importance of these aspartate residues in regulating the subnuclear localization of FANCD2, but also suggest the possibility of uncoupling the functions of FANCD2 in nuclear foci formation and DNA repair.

4. Discussion

In this study, we demonstrated that a member of the FA proteins, FANCD2, is cleaved specifically by caspase 3 at four or more sites during apoptosis. Although previous studies reported caspase-mediated regulation of FA proteins [29,30], this is the first study to identify both a specific caspase in and the cleavage sites within the FA proteins.

Following DNA damage, FANCD2 is regulated by post-translational modification [7]. Our results raise the possibility that FANCD2 is regulated through proteolytic degradation triggered by caspase 3 during DNA damage-induced apoptosis. Based on the structural data [9], all of the cleavage sites we identified are located on the outside surface of the FANCD2 protein, but not on the interface involved in the FANCI interaction, suggesting that caspase 3 can easily access these cleavage sites even in the context of the ID complex. Furthermore, cleavage products from FANCD2 (D2-XS) tended to accumulate in the presence of a proteasome inhibitor (lactacystin) (Figs. 1C and S1). Because the Z-VAD-fmk treatment prevented the appearance of D2-XS even in the presence of lactacystin, these results suggest that the FANCD2 protein is cleaved first by caspase 3 in an endolytic manner, and then further degraded by the ubiquitin–proteasome system. However, we cannot rule out an alternative model in which full-length FANCD2 is also a target for the proteasome, because the level of endogenous FANCD2 was slightly increased by lactacystin treatment (Fig. S1).

We also noticed that treatment with high doses of cisplatin induced attenuation of full-length FANCD2, even in a cell line expressing the FANCD2 4DA mutant, which does not undergo apparent cleavage (Fig. 7A). This dose-dependent attenuation of FANCD2 might arise from its proteolytic degradation via the proteasome.

Although mono-ubiquitylation of FANCD2 is critical for downstream events of the canonical FA pathway [7], caspase-mediated cleavage of FANCD2 does not depend on either the functional FA core complex (Fig. 4A) or the lysine residue at the mono-ubiquitylation site (K561; Fig. 4B). On the other hand, our results showed that functional p53 is not essential for FANCD2 cleavage, but activated p53 does promote the cleavage (Fig. 5). Another group revealed that exogenous expression of FANCD2 induces apoptosis, but not in cells lacking functional p53 [31], implicating FANCD2 in p53-dependent apoptosis. FANCD2 cleavage in p53-deficient cell lines presumably reflects the activity of the p53-independent apoptosis pathway. p53 is one of the most frequently mutated genes in human tumors; therefore, an understanding of the molecular mechanism underlying such an apoptotic pathway would be clinically relevant, because the pathway components represent potential therapeutic targets.

Amino-acid substitutions at FANCD2 cleavage sites did not interfere with mono-ubiquitylation induced by DNA damage, the ability to rescue the FANCD2-deficient cell line’s sensitivity to ICL damage, or the interaction with FANCI (Fig. 7), suggesting that cleavage per se is not required for FANCD2 functions in DRRs and/or DNA repair. Notably, however, nuclear foci formation was seriously impaired in the cell line expressing caspase-resistant FANCD2 (Figs. 7E and S3). Consistent with this, another group reported that the FANCD2–ubiquitin fusion protein was competent to mount a DDR but lacked the ability to form nuclear foci [8]. Taken together, these data suggest that nuclear foci formation by FANCD2 is involved in a process other than DNA repair.
Furthermore, we noticed that the cell line expressing the FANCD2 4DA mutant protein was slightly more resistant to MMC than the control cell line expressing wild-type FANCD2 (Fig. 7B). Other studies reported that caspase-mediated cleavage of DNA repair proteins inhibited their functions as a suppressor of apoptosis [18,22,23]. However, the 4DA mutations in FANCD2 seemed to have little, if any, effect on apoptosis induction, judging from hallmark events of apoptosis such as the appearance of cleaved PARP1 and caspases (Fig. 7D). It is possible that protection of FANCD2 from cleavage may be beneficial for cell survival with sustained DNA repair functions, although we cannot exclude the possibility that these amino-acid changes affect interactions with other proteins involved in the DDR and/or apoptosis. Alternatively, acute exposure to cisplatin may induce not only apoptosis, but also non-apoptotic cell death such as necroptosis. Further studies of FANCD2 cleavage will shed light on the possible effects of this phenomenon on DNA damage-induced cell death.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fjebslet.2014.08.027.

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