Direct DNA Binding Activity of the Fanconi Anemia D2 Protein*

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It is known that the Fanconi anemia D2 protein is vital for protecting the genome from DNA damage, but what activities this protein has are unknown. In these experiments we purified full-length Fanconi anemia protein D2 (FANCD2), and we found that FANCD2 bound to DNA with specificity for certain structures: double strand DNA ends and Holliday junctions. Proteins containing patient-derived mutations or artificial variants of the FANCD2 protein were similarly expressed and purified, and each variant bound to the Holliday junction DNA with similar affinity as did the wild-type protein. There was no single discrete domain of FANCD2 protein that bound to DNA, but rather the full-length protein was required for structure-specific DNA binding. This finding of DNA binding is the first biochemical activity identified for this key protein in the Fanconi anemia pathway.

Fanconi anemia (FA) is a disease with autosomal recessive inheritance and characterized by developmental abnormalities, progressive bone marrow failure, and cancer predisposition. Cells from FA patients exhibit hypersensitivity to DNA cross-linking agents, such as mitomycin C and cisplatin, suggesting a role for these proteins in the repair of damaged DNA. Currently, at least 11 FA complementation groups are known to exist, and the nine genes have been identified and cloned, referred to as FA subtypes A, B, C, D1/BRCA2, D2, E, F, G, and L (7–12). The encoded FA proteins cooperate in a common pathway: the FA/BRCA pathway (2). Seven of the FA proteins, A, B, C, E, F, G, and L (7–12), are known to exist, and the nine genes have been identified and cloned, referred to as FA subtypes A, B, C, D1/BRCA2, D2, E, F, G, and L (7–12). The encoded FA proteins cooperate in a common pathway: the FA/BRCA pathway (2). Seven of the FA proteins, A, B, C, E, F, G, and L, form a protein complex constitutively found in the nucleus of cells, the FA protein complex (9, 13–15). The FA complex functions to activate FANCD2 by monoubiquitinating the protein following response to DNA damage (9, 16). The activated FANCD2 protein is subsequently targeted to subnuclear foci, which are thought to be the sites of DNA repair and which also contain BRCA1, FANCD1/BRCA2, and Rad51 (2, 16–18). The deubiquitination of FANCD2, by USP19 (19), results in its inactivation and release from the sites of DNA repair. In addition, FANCD2 is regulated by phosphorylation by ATM and ATR following DNA damage to the cell (20, 21). Phosphorylation by ATM is required for an intra-S phase checkpoint response but is independent of FANCD2 monoubiquitination (20). Since the function of many of the FA proteins is to ubiquitinate and thus activate FANCD2, it seems that FANCD2 is the key effector protein in the FA pathway. While the function of FANCD2 is vital to the cell, it is unknown what biochemical activities FANCD2 may possess.

FA cells are defective in the repair of DNA double strand breaks and of interstrand cross-links (22–25). Repair of double strand DNA breaks can occur by non-homologous end joining or by homologous recombination (26–29), and FA cells have defects in both pathways (24, 25, 30, 31). FANCD1/BRCA2 has a well defined role in homologous recombination (12, 32–35). In addition, FANCD1/BRCA2 has a role in stabilizing stalled replication forks (36), suggesting that other FA proteins may be involved in this process. In both homologous recombination and collapsed replication forks, Holliday junction DNA may exist as an intermediate. We hypothesized that FANCD2 protein may bind to Holliday junction DNA as part of its function in DNA damage repair.

As a first step in the characterization of FANCD2 in the repair of DNA, we expressed and purified full-length wild-type and mutant FANCD2. We found that the unmodified protein has DNA binding activity specific for Holliday junction DNA, and FANCD2 also binds with high affinity to DNA double strand ends.

MATERIALS AND METHODS

Expression and Purification of FANCD2 Proteins—Recombinant full-length wild-type and mutant FANCD2 and wild-type FANCA were each expressed using recombinant baculovirus infected insect cells. The encoded FA proteins contained at the amino terminus a az domain, for purification on IgG-Sepharose. Tagged genes were cloned into a modified pFastBac vector (Invitrogen) and processed to generate recombinant baculovirus and used to infect S9 insect cells using standard procedures. After 72 h, the infected cells (1 × 10⁷) were harvested and washed in chilled phosphate-buffered saline. Cells were lysed in 15 ml of buffer B (20 mM Hepes, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 1% protease inhibitor mixture (Sigma)). The pre-equilibrated 250 μl of IgG-Sepharose (Amersham Biosciences) was added into the protein lysate and incubated with mixing at 4 °C for 1 h. The protein-IgG-Sepharose complexes were washed twice with buffer B, two times with buffer C (20 mM Hepes, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 1% protease inhibitor mixture (Sigma)), four times with TEV cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 0.5 mM dithiothreitol), four times with TEV cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol), four times with TEV cleavage buffer plus 18 μg of TEV enzyme. Beads containing the bound
fusion protein were incubated with the TEV protease at 4 °C for 16 h. The supernatant contained FANCD2 protein and TEV protease. After elution, each FANCD2 or FANCA protein had appended to its amino terminus nine amino acids derived from the expression construct: Ser-Gly-Thr-Val-Asp-Gly-Ala-Gly-Gln. Details of the baculoviral vector are available from the authors.

FANCD2 fragments containing amino acid residues 1–989, 1–472, 473–989, and 945–1451 were expressed in *Escherichia coli*. The cDNA for each FANCD2 fragment was tagged at the 3′-end, replacing the stop codon with a hexahistidine tag (pET-21a(+) vector; Novagen), and the fusion proteins were expressed in Rosetta(DE3)pLysS cells (Novagen) using standard procedures. The FANCD2 fragments were purified on Ni2+-nitrilotriacetic acid-agarose resin (Qiagen), followed by a purification step using a Bio-Scale Q column (Bio-Rad). The NH2-terminal domain (35–339 amino acids) of FANCD2 was expressed as a glutathione S-transferase fusion, using standard procedures. The concentrations of DNA ends in competitor DNAs were: 25 nM (lane 3), 200 nM (lane 4), 0.3 nM (lane 7), 2.5 nM (lane 8), 3 nM (lane 9), and 24 nM (lane 10).
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**RESULTS**

**FANCD2 Binds to DNA—**Since FANCD2 functions in the nucleus and regulates the repair of DNA damage (2, 16, 17), we tested whether the FANCD2 protein binds to DNA. The full-length human FANCD2 protein was expressed in insect cells and purified via an associated zz-domain affinity tag. Full-length FANCD2 was released from the matrix after cleavage by the TEV protease at a site at the amino terminus. By Coomassie staining, greater than 90% of the protein detected migrated at about 160 kDa, consistent with full-length, unmodified FANCD2 (Fig. 1A, left). The concentration of the FANCD2 protein was determined by comparing the intensity of the band in lane 4 to quantitation standards on the same gel (data not shown). A typical protein preparation from 108 Sf9 cells contained 0.5 nmol (90 μg) of FANCD2 protein at a concentration of 1.7 μM. The 160-kDa band was confirmed to be FANCD2 by immunoblot using an antibody specific for FANCD2 (Fig. 1A, right).

We hypothesized that the FANCD2 protein binds to DNA in a structure-specific fashion. Since Holliday junction DNA is a potential intermediate in the homologous recombination pathway, we tested this structure first. In an electrophoretic mobility shift assay (EMSA), full-length FANCD2 retarded the mobility of a 125-bp Holliday junction DNA probe (four 30-bp arms; Fig. 1B, lane 3). Three different recombinant protein preparations from baculovirus-infected cells were analyzed. FANCD2 was compared with FANCA and to a mock purification from empty baculovirus-infected cells. FANCD2 (40 nM) bound to the Holliday junction DNA and migrated in a diffuse shift that formed a band below the origin of the gel. FANCA protein did not bind to the Holliday junction DNA probe at the same concentration of FANCD2 protein (Fig. 1B, lane 2). The shifted bands in the reaction with FANCA (lane 2) were also present in the mock purification from the cells infected with empty virus (lane 1), indicating that these complexes were low level, nonspecific contaminants.

FANCD2 was included in DNA binding reactions at different concentrations (Fig. 2A). We observed diffuse bands that migrated progressively slower in reactions with higher FANCD2 concentrations. This finding was most consistent with the interpretation that the protein-DNA complex contained multiple FANCD2 molecules. Since the stoichiometry of DNA binding could not be determined, we did not determine a $K_d$ for the complex. Instead, we estimated binding affinity by the concentration of FANCD2 protein that bound half of the probe. Approximately half of the Holliday junction DNA probe was bound in reactions containing 15 nM FANCD2 (Figs. 2A and 4B).

The specificity of DNA binding was determined using various probes and DNA competitors. FANCD2 bound to 65-bp double-stranded linear DNA (Fig. 2A) and to a 65-bp Y-shaped DNA (data not shown). When using a 65-bp linear DNA probe, 50% of the probe was bound at about 20 nM FANCD2, indicating that the binding affinity of FANCD2 to linear 65 bp was modestly decreased relative to the 125-bp Holliday junction probe. As with the Holliday junction probe, the FANCD2-bound DNA formed a diffuse band that had slowed migration as more protein was included in reactions. At the highest protein concentrations tested, the FANCD2-linear DNA complex migrated just beneath the origin of the gel (Fig. 2A, lanes 7 and 8). As in the case with the Holliday junction probe (Fig. 2A, lanes 1–4), we interpreted these results with the 65-bp linear probe to indicate that multiple FANCD2 molecules bound to the DNA probe.

We tested the specificity of FANCD2 for different forms of DNA using competition reactions. The probe in each reaction was 1 ng of the 125-bp Holliday junction DNA. A 5-fold excess of self-competitor resulted in a 35% reduction of the bound probe (Fig. 2B, lane 3). A 40-fold excess of self-competitor resulted in near complete competition (lane 4). Supercoiled plasmid DNA competed modestly with the Holliday junction probe for binding to FANCD2 (Fig. 2B, lanes 5 and 6). By contrast, when this same plasmid was cleaved at a single site generating an ~5000-bp linear DNA, the level of competition was similar to the self-competition (Fig. 2B, lanes 7 and 8). Since the principal difference between the supercoiled plasmid and the linear DNA was the presence of DNA ends, we tested whether a higher concentration of DNA ends bound to FANCD2 with higher affinity. Cutting this plasmid at a total of nine sites (average size of the linear DNA was ~500 bp) made this dsDNA the most efficient competitor tested for FANCD2 binding. Nearly complete competition for the Holliday junction probe was observed with a 5-fold excess of the 500-bp linear DNA.
DNA (Fig. 2B, lane 9). In lanes 5–10, the competitor DNAs were all derived from the same plasmid with the only difference being the number of ends: 0, 2, or 18. These results supported the notion that the FANCD2 binds to DNA ends.

In most of the experiments in Figs. 1 and 2, the probe was Holliday junction DNA. FANCD2 binding to the Holliday junction DNA could be due to binding to ends (four ends per molecule) or due to binding to the internal part of the Holliday junction. We directly compared which of these DNA structures bound to FANCD2 with higher specificity. The competitor DNAs were normalized by the moles of DNA ends of unlabeled Holliday junction DNA or 65-bp linear duplex DNA included in reactions with Holliday junction probe. The linear duplex DNA was equivalent in length to two arms of the Holliday junction probe. In this experiment, 1 ng of Holliday junction probe was used; thus, the concentration of the probe was 1.2 nM, but since it had four ends, the concentration of dsDNA ends was about 5 nM. Significant competition for DNA binding by FANCD2 was apparent when reactions contained 25 nM Holliday junction ends (5 ng DNA; Fig. 3, lane 3), but there was very little competition for binding when reactions contained 200 nM 65-bp duplex DNA ends (lane 10). This result suggested that for DNAs of similar size, FANCD2 bound to the Holliday junction DNA with higher affinity than it bound to DNA ends. An alternative model for the specificity was that the ends of the Holliday junction DNA were closely juxtaposed and favored binding of the FANCD2 when compared with the linear DNA. Since FANCD2 bound to both, DNA ends and Holliday junctions, the specificity of the DNA binding activity was consistent with a role for FANCD2 in the repair of double-stranded DNA damage.

Not all linear DNA molecules were equivalent for binding to FANCD2. When comparing the competition for binding to the Holliday junction probe, the linear 500-bp DNA was the most effective competitor (complete competition with about 3 nM ends; Fig. 2B, lane 9), while the 65-bp linear DNA did not compete (Fig. 3, lanes 7–10). Since the protein-DNA complex was observed as a diffuse band that migrated with progressively slower speed as more FANCD2 was included in reactions, we infer that FANCD2 binds to DNA as a multimer. The competition data were most compatible with the model that the FANCD2 nucleated its binding on the DNA end, and the FANCD2 then spread along the length of the DNA. Thus, when comparing the three linear DNAs tested for binding to FANCD2, the 5000-bp linear DNA had too low a concentration of ends, and the 65-bp linear DNA was too short, but the 500-bp linear DNA had the best balance of DNA ends and length.

**FANCD2 Mutant and Wild-type Proteins Bound to the DNA with Similar Affinities**—The carboxyl terminus of the FANCD2, encoded by exon 44, is highly conserved among
homologues in other eukaryotic species, and therefore this domain may have an important cellular function required for mitomycin C resistance (11). This domain is highly acidic, containing 12 acidic residues out of 24 residues and is referred to as the “EDGE” domain. We purified a FANCD2 mutant, in which the first aspartic acid residue encoded in exon 44 was converted to alanine (D1428A). In addition, we truncated the acidic domain of FANCD2 (E44t) by expressing a fusion protein, which encodes no exon 44. Complementation studies reveal that expression of these mutants in FANCD2-deficient cells does not correct the FA phenotype (39). A Fanconi anemia patient-derived mutation, which has no exon 17 (11), was also expressed and purified (E17del). All three mutant FANCD2 proteins were expressed and purified similarly as was the wild-type protein (Fig. 4A). Each of these protein preparations was analyzed for binding to Holliday junction DNA (Fig. 4B). Each mutant form of FANCD2 bound to the DNA with an affinity similar to wild-type protein. Removal of an acidic domain, as in the E44t FANCD2, would be predicted to increase affinity of a protein for DNA, but clearly, the magnitude of the effect was very small. The FANCD2 mutant D1428A did bind less avidly to the probe. While wild-type, E44t, and E17del FANCD2 each bound to 50% of the probe at about 40 nM, FANCD2 mutant D1428A did bind less avidly to the probe. While wild-type, E44t, and E17del FANCD2 each bound to 50% of the Holliday junction probe at about 40 nM (Fig. 4B). This decrease in affinity of the mutant FANCD2-D1428A for binding DNA was small and not seen with the E44t truncated protein, in which D1428 was deleted. Furthermore, the patterns of the bands on the gel were similar when comparing the D1428A mutant with the wild-type FANCD2. We thus conclude that these mutations, which are associated with a Fanconi anemia phenotype (39), do not significantly affect DNA binding properties of the FANCD2 protein.

In an effort to map the DNA binding domain of FANCD2, we expressed and purified fragments of the protein. Data from Fig. 4 revealed that a 44-amino acid domain from 472–515 was dispensable for DNA binding in the E17del mutant, and the 24-amino acid deletion from 1427–1451 in the E44t mutant was dispensable for DNA binding in the E44t mutant was also competent for binding Holliday junction DNA. We expressed a number of FANCD2 protein fragments, but in all cases the level of expression was low, and many of the different fragments were insoluble under nondenaturing conditions (data not shown). We did succeed in purifying the following FANCD2 fragments: 35–339 fused to glutathione S-transferase, 1–989, 1–472, 473–989, and 945–1451. The protein fragments containing sequences 1–989, 1–472, and 473–989 all bound to the Holliday junction probes at a lower concentration than did the full length (supplemental Fig. S2), suggesting an increased affinity for the DNA. However, competition analysis revealed that all FANCD2 fragments bound to DNA in a nonspecific fashion (supplemental Fig. S2). No identifiable DNA binding motif is revealed from the protein sequence. We conclude from these analyses that, with the exception of small deletions as in Fig. 4, nearly the full length of FANCD2 is required for DNA binding activity.

DISCUSSION

The phenotype of Fanconi anemia cells indicates that the FA pathway is critical to the repair of DNA damage. Cell lines harboring mutations in the FA pathway are defective for homologous recombination (30) and in stabilizing stalled replication forks (36). In both of these processes, Holliday junctions may occur, and if there is DNA damage, dsDNA ends also occur. Published results suggest that the FANCD2 protein is a key effecter protein in the double strand break repair pathway (2, 16, 17, 30, 40). However, all results to date are based on the function of Fanconi anemia proteins in cells, and the biochemical activities of many of the FA proteins, which contribute to the phenotype, are unknown. In the current study, we purified full-length unmodified FANCD2 protein to analyze the function of FANCD2 in an assay relevant to the repair of DNA damage. We found that the FANCD2 protein binds to DNA with specificity for certain structures: double strand DNA ends and Holliday junctions. We found that there is no single discrete domain in the FANCD2 protein that binds to DNA, but rather the full-length protein is required for structure-specific DNA binding. Interestingly, binding to dsDNA ends also required sufficient length of the DNA, greater than 65 bp. We suggest that the FANCD2-DNA complex nucleates by binding to the end, followed by multiple other FANCD2 molecules binding along the length of the DNA to form the FANCD2-DNA complex. Clearly, further work is required to understand how this DNA binding activity promotes repair of DNA lesions.

In cells, following DNA damage, the FANCD2 protein is phosphorylated and monoubiquitinated, and the modified FANCD2 becomes tightly associated with chromatin and localized in DNA repair foci (2, 16, 17, 39). In this study, we found that the unmodified FANCD2 bound to DNA. We thus propose that modification of FANCD2 by ubiquitination and phosphorylation would likely increase its affinity for DNA, particularly at sites of DNA damage. Alternatively, the ubiquitin moiety appended to FANCD2 may promote high affinity binding, via an ubiquitin-binding domain, to another chromatin-associated factor at sites of DNA damage. Clarification of how ubiquitination of FANCD2 modifies the DNA binding activity will be important to determine.

Most patient-associated mutations in FANCD2 result in loss of the protein (11). One FA mutation results in stable protein missing amino acid residues 472–515 due to splicing out of exon 17 (11). In addition, the acidic carboxyl-terminal EDGE domain has been implicated in the proper function of FANCD2 in cells (39). We found that the mutant FANCD2 proteins bound to the DNA with only marginal differences in affinity from the wild type. While we have identified a biochemical activity of FANCD2, this function is not the activity affected by disease-causing point mutations.

In summary, the findings from this study reveal that the FANCD2 protein binds to Holliday junction DNA and to DNA ends. This is the first biochemical activity identified for this key protein in the Fanconi anemia pathway. This activity is consistent with a role for the FANCD2 protein in the repair of double-stranded DNA breaks. However, it is unclear how phosphorylation and ubiquitination of FANCD2 regulate this activity, and more work will be required to determine how the FANCD2 protein functions in the repair of DNA damage.

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