Specialization among Iron-Sulfur Cluster Helicases to Resolve G-quadruplex DNA Structures That Threaten Genomic Stability*

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**Background:** The Fe-S helicase FANCJ implicated in Fanconi anemia plays important roles in DNA replication and repair.

**Results:** FANCJ, but not the Fe-S XPD or DDX11 helicases, unwinds unimolecular G4 DNA.

**Conclusion:** FANCJ is a specialized Fe-S helicase, preventing G4-induced DNA damage.

**Significance:** FANCJ has a unique role in DNA metabolism to prevent G4 accumulation that causes genomic instability.

G-quadruplex (G4) DNA, an alternate structure formed by Hoogsteen hydrogen bonds between guanines in G-rich sequences, threatens genomic stability by perturbing normal DNA transactions including replication, repair, and transcription. A variety of G4 topologies (intra- and intermolecular) can form in vitro, but the molecular architecture and cellular factors influencing G4 landscape in vivo are not clear. Helicases that unwind structured DNA molecules are emerging as an important class of G4-resolving enzymes. The BRCA1-associated FANCJ helicase is among those helicases able to unwind G4 DNA in vitro, and FANCJ mutations are associated with breast cancer and linked to Fanconi anemia. FANCJ belongs to a conserved iron-sulfur (FeS) cluster family of helicases important for genomic stability including XPD (nucleotide excision repair), DDX11 (sister chromatid cohesion), and RTEL (telomere metabolism), genetically linked to xeroderma pigmentosum/Cockayne syndrome, Warsaw breakage syndrome, and dyskeratosis congenita, respectively. To elucidate the role of FANCJ in genomic stability, its molecular functions in G4 metabolism were examined. FANCJ efficiently unwound in a kinetic and ATPase-dependent manner entropically favored unimolecular G4 DNA, whereas other Fe-S helicases tested did not. The G4-specific ligands Phen-DC3 or Phen-DC6 inhibited FANCJ helicase on unimolecular G4 ~1000-fold better than bi- or tetramolecular G4 DNA. The G4 ligand telomestatin induced DNA damage in human cells deficient in FANCJ but not DDX11 or XPD. These findings suggest FANCJ is a specialized Fe-S cluster helicase that preserves chromosomal stability by unwinding unimolecular G4 DNA likely to form in transiently unwound single-stranded genomic regions.

DNA sequences that form alternate DNA structures such as hairpins, triplexes, Z-DNA, and G-quadruplexes (G4) can be difficult to replicate and pose a source of genomic instability. In addition, they may have effects on transcription, recombination, or DNA repair. Increasing evidence suggests that G4 structures indeed form in vivo and have readily detectable effects on cellular nucleic acid metabolism (1). G-quadruplexes are composed of planar stacks of four guanine residues interacting with each other by noncovalent Hoogsteen hydrogen bonds and stabilized by a monovalent cation (typically Na⁺ or K⁺) residing in the central cavity. G-quadruplexes, which have the ability to self-assemble in G-rich sequences of the genome, can assume different topologies (uni-, bi-, or tetramolecular). A growing number of mammalian DNA helicases (e.g. WRN, BLM, FANCJ, PIF1) have been shown to unwind G4 DNA substrates in vitro; however, not all DNA helicases are able to do so efficiently (e.g. RECQ1) or remain uncharacterized at the biochemical level (e.g. RTEL) (2).

It was only recently that cellular evidence was obtained demonstrating that the ability of certain DNA helicases to resolve G-quadruplexes in vitro had any relevance in vivo. A role of *Saccharomyces cerevisiae* Pif1 to resolve G4 structures to preserve replication fork progression and suppress chromosomal breakage was determined (3). Inactivation of Pif1, but not the RecQ homolog Sgs1, destabilized a G-rich human subtelomeric CEB1 mini-satellite inserted into a yeast chromosome (4). Treatment of Pif1-deficient cells with G4 binding compounds exacerbated CEB1 instability but not tandem repeats lacking G4-forming sequences (5). The hCEB1 array is unstable in Pif1-deficient cells or yeast exposed to the G4 binding compound Phen-DC6 only when the G-rich strand is the template of leading-strand replication (6). This result was not entirely predictable as the lagging strand template has more extensive single-
FANCJ is a Specialized Fe-S Helicase in G4 DNA Metabolism

stranded character as a consequence of discontinuous DNA synthesis. Earlier work had suggested that deletions in G-rich sequences of the Caenorhabditis elegans genome due to the mutation of the Fe-S helicase DOG-1 was the consequence of structural blocks (secondary structures) to lagging strand synthesis (7). The findings that G4-induced instability can occur during leading or lagging strand replication raises the question of how the division of labor between G4-resolving DNA helicases occurs and potential differences in substrate specificity or underlying mechanism.

Human cells depleted of the Fe-S cluster helicase FANCJ, a DOG-1 ortholog, were found to be sensitive to the G4-specific DNA binding compound telomestatin (TMS) (8), previously shown to cause telomere instability (9, 10). Patient-derived FANCJ mutant cells accumulate deletions at genomic sequences with a G4 DNA signature (11). These findings suggested a model in which FANCJ suppresses genomic instability by resolving G-quadruplexes to enable smooth replication fork progression. Transcription profiling of chicken DT40 mutants suggested that FANCJ maintains epigenetic stability at G4 DNA motifs by collaborating independently with the REV1 translesion polymerase or the RecQ helicases WRN and BLM defective in the genetic disorders Werner syndrome and Bloom syndrome, respectively (12). More recently, it was reported that in chicken cells, FANCJ promotes replication through TMS-stabilized DNA sequences predicted to form G4 and suppresses heterochromatin spreading (13).

The cumulative evidence implicates FANCJ in G4 biology; however, it is still unclear how FANCJ is unique from other DNA helicases, including those that share sequence homology in the helicase core domain. In the current study our efforts were focused on the Fe-S family of DNA helicases and their potential differences in substrate specificity based on G4 topology. FANCJ was determined to be unique among the Fe-S helicases tested for its ability to unwind unimolecular G4 DNA. The G4 ligand TMS induced DNA damage in human cells in a manner dependent on the status of FANCJ but not the Fe-S DNA helicases DDX11 or XPD. These results suggest that FANCJ is specialized among the Fe-S helicases to resolve G4 DNA structures that threaten genomic stability.

**EXPERIMENTAL PROCEDURES**

Recombinant Proteins—Recombinant human FANCJ (14), DDX11 (ChlR1) (15), and Thermoplasma acidophilum XPD (16) proteins were purified as described. For expression and purification of Escherichia coli DinG, the entire DinG open reading frame was inserted into the pETM11 (EMBL) plasmid with sequence encoding His-tag resulting in pDING1. pDING1 was transformed into E. coli BL21 (DE3) cells. Expression was induced by 0.1 mM isopropyl 1-thio-β-d-galactopyranoside when the bacterial culture reached an A 600 of 0.6 and incubated at 15 °C for 15 h. Cells were harvested and lysed in 50 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 1 mM NaCl, 0.1% Triton X-100, 30% glycerol, protease inhibitor (Roche Applied Science), and DNase (Invitrogen). DinG was purified by affinity chromatography using nickel IDA beads (Macherey & Nagel). DinG protein was eluted in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 1 mM NaCl, 10% glycerol, and 250 mM imidazole. For further purification, a size exclusion chromatography step was applied using 20 mM Tris-HCl (pH 8.0), 1 mM NaCl, 1 mM DTT, and 10% glycerol as the buffer system. Finally, the protein was concentrated to concentrations between 5 and 10 mg/ml using a Centricon filter with 30-kDa cutoff (Sartorius).

DNA Substrates—The four-stranded (tetramolecular) parallel TP-G4 substrate and two-stranded (bimolecular) anti-parallel OX-1-G2 DNA substrate were prepared from gel-purified oligonucleotides purchased from Loftstrand Labs (Gaithersburg, MD) as previously described (8). The unimolecular Poly(A) Zic1-G4 substrate with a 5′ single-stranded DNA tail was prepared as previously described (17). The 19-base pair (bp) forked duplex DNA substrate was prepared from DC26 and TSTEM25 as described earlier (18). DNA substrates and oligonucleotide sequences are shown in Table 1.

Helicase Assays—Standard helicase reaction mixtures (20 μl) containing 5 fmol of the indicated TP-G4 or OX-1-G2 DNA substrates or forked duplex DNA substrate (0.25 mM DNA substrate concentration), 1 mM ATP, and the indicated concentrations of the specified helicase were as previously described for FANCJ (8) and DDX11 (15). Helicase reactions for XPD and DinG were performed in buffer that contained 20 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM dithiothreitol, 5 mM MgCl2, and 100 μg/ml BSA. Reactions were performed at 37 °C for 15 min in the presence of 1 mM ATP, the DNA substrates mentioned above, and the indicated helicase protein concentrations. For the unimolecular Poly(A) Zic1-G4 DNA substrate, helicase reactions were performed in reaction buffer containing a 20-fold excess of peptide-nucleic acid complementary oligonucleotide (17). G4 helicase reactions were terminated by the addition of 20 μl of stop buffer (74% glycerol, 0.01% xylene cyanol, 0.01% bromphenol blue, 10 mM KCl, 20 mM EDTA). Forked-duplex helicase reactions were terminated as described previously (18). Reaction products were resolved on nondenaturing 8 and 12% (19:1 acrylamide-bisacrylamide) polyacrylamide gels for the G4 and forked-duplex substrates, respectively, and quantitated as described previously (18). Briefly, the percentage of helicase substrate unwound was calculated using the formula: % unwinding = 100 × (P(S + P)), where P is the product and S is the substrate. The values of P and S have been corrected after subtracting background values in the no enzyme and heat-denatured substrate controls, respectively. Helicase data represent the means of at least three independent experiments with S.D. values shown by error bars.

G4 DNA Binding Ligands—TmPyP4 (meso-5,10,15,20-tetraakis-(N-methyl-4-pyridyl)porphine, tetratosylate) was from Calbiochem. Phen-DCp, Phen-DCp (5, 19), and TMS (9) were prepared as described previously. Compounds were dissolved in dimethyl sulfoxide. For helicase assay, various concentrations of ligands as indicated were added to the reaction mixture containing DNA substrate on ice before the addition of helicase.

Fluorescence-induced Displacement Assay—A schematic principle of the fluorescence-induced displacement (FID) assay is shown in Fig. 7A (20). Briefly, G4 substrate (OX-1-G2′, 3′-Poly(A) Zic1-G4, or 5′-Poly(A) Zic1-G4) was labeled with thiazole orange (TO), enabling the DNA substrate-TO com-
plex to fluoresce. TO displacement by the G4 ligand (Phen-DC$_3$ or Phen-DC$_9$) results in a decrease in fluorescence.

To label the G4 substrate with TO, fluorimetric titrations were performed in a 1-ml quartz cell in cacodylate buffer/Li$^+$-K$^+$ buffer in a total volume of 1 ml. The temperature (20 °C) was kept constant with a thermostatted cell holder. Titrations were performed with a solution of the fluorescent probe (TO 0.5 μM) in the corresponding buffer in which the gradual addition of oligonucleotides was carried out (up to 10 mol eq). After each addition, a fluorescence emission spectrum was recorded with excitation wavelength fixed at 495 nm. The fluorescence emission area was measured between 510 and 750 nm with 1.0-nm increments, 0.1-s integration times, and (excitation/emission) 10/10-nm slits. The titration curves were obtained by plotting the fluorescence emission area enhancement against the oligonucleotide concentration.

To perform the G4-FID experiments, the TO-labeled G4 substrates were titrated with increasing concentrations of Phen-DC$_3$ or Phen-DC$_9$, and the fluorescence decrease upon TO displacement was indicative of G4 ligand binding. Each experiment was performed in a 1-ml cell in 10 mM lithium cacodylate buffer (pH 7.4) with 100 mM KCl in a total volume of 1 ml. The fluorescence emission area was measured between 510 and 750 nm with 1.0-nm increments, 0.1-s integration times, and (excitation/emission) 10/10-nm slits. The titration curves were obtained by plotting the fluorescence emission area enhancement against the oligonucleotide concentration.

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second transfection was performed similarly. Seventy-two hours after the initial transfection, cells were harvested or treated as indicated below. Cell lysate preparation and Western blot analyses were performed as described previously (24).

DDX11 was depleted from U2 OS cells by siRNA transfection using Lipofectamine 2000 with two DDX11 siRNA sequences (5’-UCCUCGCAUGGCUAGGCAAGGCUUU-3’ and 5’-GUUCGUCGCUUUCCUCUGCGAA-3’) as described previously (25). Due to the low abundance of DDX11 in human cells, DDX11 immunoprecipitation from whole cell lysates of cells transfected with control siRNA or DDX11 siRNA was performed, and immunoprecipitated DDX11 protein was detected by Western blot. Briefly, whole cell lysates (400 μg of protein) were incubated in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 5 mM EDTA, 1× Pierce protease phosphatase inhibitor) with 2 μg of rabbit DDX11 antibody (Santa Cruz). Rabbit DDX11 antibody was preincubated with protein AG magnetic beads (Thermo Scientific) and washed, and antibody-bound protein AG beads were used for binding with whole cell lysates. Binding was allowed for 2 h at 4 °C and washed twice with lysis buffer, and bound proteins were separated in a magnetic stand. Bound proteins were boiled in SDS sample buffer and resolved by SDS-PAGE using 4–12% acrylamide gels. DDX11 was detected by Western blot using a 1:250 dilution of mouse DDX11 antibody (Sigma).

**Immunofluorescence Experiments**—Seventy-two hours after the first siRNA transfection, U2 OS cells were treated with either 5 μM TMS or 100 nM mitomycin C (MMC) for 3 h, subsequently washed with phosphate-buffered saline (PBS), returned to complete medium for 16 h, and fixed with paraformaldehyde (3.7%) at room temperature for 15 min. Fixed cells were washed twice with PBS and treated with permeabilization buffer (0.5% Triton solution (Sigma), 1% BSA, 0.2 mg/ml EDTA, and 100 mM glycine) on ice for 10 min. Cells were then washed twice with PBS and treated for 100 ng/ml RNase A for 30 min at 37 °C. Cells were then washed with PBS and blocked with 10% goat serum (Sigma) for 2 h at room temperature. Indirect immunostaining was performed by first incubating cells with a mouse anti-γ-H2AX monoclonal antibody (1:1000; Upstate) for 1 h at room temperature. After 4 washes in PBS containing 0.1% Tween 20, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (1:1000; Invitrogen) for 1 h at room temperature. Cells were washed 4 times with PBS containing 0.1% Tween 20 and coated with Prolong Gold antifade reagent containing DAPI (4’,6’-diamidino-2-phenylindole; Invitrogen). Coverslips were placed on chamber slides, and cells were cured at room temperature in the dark for 24 h. Immunofluorescence analyses were performed with a Zeiss LSM 510 META inverted Axiovert 200M laser scan microscope. Images were captured with a charge-coupled device camera and digitally processed using Adobe Photoshop CS6 to adjust brightness and contrast.

The XPD−/− and XPD+/+ cell lines were treated with 5 μM TMS for 3 h or UV light (2.5 J/m²) followed by fixation, permeabilization, and processing for γ-H2AX immunofluorescence as described above for the U2 OS cells.

**RESULTS**

**FANCJ Efficiently Unwinds Unimolecular G4 DNA**—To gain a better understanding of the FANCJ role in G4 DNA metabolism, we examined its potential uniqueness from other Fe-S DNA helicases implicated in DNA repair processes that might involve G-quadruplex DNA structures. Because G-quadruplexes are known to assume different topologies based on Hoogsteen hydrogen bonding between G-rich strands in distinct orientations, we tested FANCJ to unwind an entropically favored unimolecular G4 structure and compared its activity to multistranded G4 substrates that have been more conventionally used to evaluate DNA unwinding by various DNA helicases in vitro.

FANCJ unwound the unimolecular Poly(A) Zic1-G4 DNA substrate in the presence of ATP in a kinetic manner to near completion by the end of the 45-min incubation as evident by the co-migration of the unwound product with the radiolabeled duplex marker (Fig. 1A). Quantitative assessment of FANCJ unwinding of the unimolecular G4 substrate during the first 15 min was used to determine its initial linear rate of 10 pm unimolecular G4 substrate unwound per min under multturnover conditions (Fig. 1B). FANCJ failed to unwind the unimolecular G4 substrate in the absence of ATP or in the presence of ADP or ATPγS (Fig. 1C), indicating that FANCJ helicase activity on the unimolecular G4 substrate required nucleotide hydrolysis. FANCJ bound the unimolecular G4 substrate irrespective of nucleotide (data not shown), indicating that its inability to unwind was not attributed to its inability to interact with the G4 substrate.

To determine if the observed helicase activity on the unimolecular G4 substrate was dependent on ATP hydrolysis intrinsic to FANCJ, we tested an engineered FANCJ ATPase-dead mutant (K52R) purified in a manner identical to that of the normal FANCJ recombinant protein for unwinding Poly(A) Zic1-G4. The K52R mutant protein failed to resolve the G4 substrate (Fig. 1D), confirming that FANCJ helicase activity on the unimolecular G4 substrate is dependent on FANCJ ATP hydrolysis. Similarly, a patient-derived FANCJ-A349P mutant, which inactivates FANCJ helicase activity on conventional forked duplex and multistranded G4 substrates but not its ability to hydrolyze ATP (8), disabled FANCJ helicase activity on the unimolecular G4 substrate (Fig. 1D).

We next examined FANCJ helicase activity on the unimolecular G4 substrate as a function of protein concentration. FANCJ unwound the unimolecular G4 (Fig. 2A), forked duplex (Fig. 2B), four-stranded G4 (Fig. 2C), and two-stranded G4 substrates (Fig. 2D) in a FANCJ concentration-dependent manner. Interestingly, a significantly greater percentage of the unimolecular G4 substrate was unwound compared with the four-stranded G4 substrate or the 19-bp forked duplex DNA substrate at FANCJ concentrations below the enzyme saturating substrate at FANCJ concentrations below the enzyme saturating plateau in which ~95% substrate was unwound by FANCJ (Fig. 2E). However, the two-stranded G4 substrate was unwound better by FANCJ compared with the unimolecular G4 substrate at subsaturating enzyme concentrations. It should be kept in mind that the number of G-tetrads and loop length would be expected to influence G-quadruplex stability (26). The conser-
sus sequence for quadruplex formation currently used in bioinformatics predictions is \((G_{3-N}N_{1-7})_4\), where \(G\) stands for runs of guanines, and \(N\) stands for any base in the loop. According to this, we expect the Poly(A) Zic1-G4 DNA substrate to form a 3-tetrad quadruplex, whereas the bimolecular OX-1-G2’ to form a 4-tetrad quadruplex. In this regard the resulting quadruplex folds of the G4 substrates tested are not expected to be greatly different in stability; however, loop length may also influence topology and stability (27). Therefore, prediction of G4 stability is complex and an active area of interest (28). The tetramolecular TP-G4 escapes the consensus used for bioinformatics and would be expected to form a more stable G-quadruplex with as many as 14-tetrads possible.

In addition to the effects of tetrad number and loops on G4 stability, the number of 5’ single-stranded DNA overhangs available for FANCJ helicase to load onto the G4 substrate is a factor that may influence unwinding activity. The tetramolecular TP-G4 substrate can bind as many as four helicase molecules, although steric constraints may not allow each strand to be bound. The bimolecular OX-1-G2’ substrate may be expected to accommodate two helicase molecules. The number of 5’ overhangs for FANCJ to load may contribute to the greater unwinding of the bimolecular versus unimolecular G4 substrate.

**Fe-S Cluster DNA Helicases DDX11 or DinG Fail to Unwind Unimolecular G4 DNA**—The preference of FANCJ to unwind the unimolecular G4 substrate led us to ask if other Fe-S cluster DNA helicases might behave similarly or different from FANCJ. We first tested purified recombinant human DDX11, the helicase protein that is defective in Warsaw Breakage syndrome (29, 30). DDX11 was unable to unwind the unimolecular Poly(A) Zic1-G4 substrate (Fig. 3A) that was efficiently unwound by FANCJ. Under these reaction conditions, DDX11 efficiently unwound forked duplex DNA (Fig. 3B). Neither greater concentrations of DDX11 or an increased time of incubation increased DDX11 helicase activity over background (<3%) for the Poly(A) Zic1 G4 substrate (data not shown). DDX11 poorly unwound the tetramolecular G4 DNA substrate (Fig. 3C) but was able to unwind the bimolecular OX-1-G2’ (Fig. 2D), consistent with previous results (15), although not nearly as well as forked duplex substrate (Fig. 3E). These results demonstrate that the unimolecular G4 substrate preference of FANCJ is distinct from that of the sequence-related Fe-S helicase DDX11.

We next tested the *E. coli* Fe-S DNA helicase DinG for its activity on uni-, bi-, and tetramolecular G4 substrates and the forked duplex substrate. DinG failed to unwind the unimolecular G4 substrate (Fig. 4A) under conditions that it efficiently unwound the forked duplex to near completion (Fig. 4B). Neither increased DinG protein concentration or incubation time resulted in any increased unwinding of the unimolecular Poly(A) Zic1 DNA substrate over background (data not shown). In contrast to its poor activity on unimolecular G4 DNA, DinG unwound the four-stranded TP-G4 substrate in a
protein concentration-dependent manner nearly as efficiently as forked duplex (Fig. 4, C and E). The two-strand OX-1-G2’ substrate was also unwound by DinG, achieving 70% substrate unwound by 2 nM DinG (Fig. 4D). However, it was evident from inspection of the helicase data that DinG was less active on the four-stranded G-quadruplex compared with the two-stranded G4 or forked duplex (Fig. 4E).

**XPD Helicase Fails to Unwind all Three Topological G4 DNA Structures**—Given the apparent differences in DNA unwinding among the Fe-S helicases FANCJ, DDX11, and DinG for the various G4 substrates, we next tested purified recombinant XPD protein from *T. acidophilum* for its helicase activity. *T. acidophilum* XPD helicase was unable to unwind uni-, bi-, or tetramolecular G4 substrates under conditions that it unwound the forked duplex substrate to near completion (Fig. 5). Based on these results and those from testing the other Fe-S DNA helicases, we conclude that the robust activity of FANCJ to unwind unimolecular G4 DNA is distinct from that of XPD and the other Fe-S DNA helicases.

**Variation in Potency of G4 Ligands to Inhibit G4 Helicase Activity in a Manner Dependent on G4 Topology**—There has been growing interest in compounds that specifically bind G4 DNA and their potential application for medicinal chemistry such as chemotherapy drugs. For example, the G4 ligand TMS is known to induce telomere capping defects by interfering with telomerase activity (10) and perturbing the interaction of shelterin proteins with telomeres (31). It was proposed that the effect of G4 ligands to destabilize telomeres may be used as an anti-tumor strategy to cause cancer cells to senesce. Given the interest in G4 ligands and their mechanism of action, we wanted to evaluate if such molecules were able to inhibit helicase-catalyzed unwinding of G4 structures that vary in their topology.

We began by testing the effect of G4 ligands including the porphyrin TMPyP4, TMS, and two Phen-DC bisquinolinium compounds on FANCJ unwinding of a unimolecular Poly(A) Zic1 G4 substrate. TMS and Phen-DC3 inhibited FANCJ unwinding of the unimolecular G4 substrate in a drug concentration-dependent manner (Fig. 6). Inhibition of FANCJ helicase activity by either TMS or Phen-DC3 was specific to G4 DNA structures because little to no effect of the drug on FANCJ unwinding of a forked duplex DNA substrate was observed (data not shown). The 50% inhibitory concentrations (IC50 values) of TMS were very similar for the uni-, bi-, and tetramolecular G4 substrates tested (IC50 ~ 2 nM) and comparable with those values previously reported for TMS inhibition of FANCJ helicase activity on various multistranded G4 substrates (8) (Table 2). The G4 ligand TMPyP4 was also able to inhibit FANCJ helicase activity on all three G4 substrates; however, its
effect was very modest as demonstrated by the large IC_{50} values. In contrast, Phen-DC3 was a more potent inhibitor of FANCJ G4 helicase activity on the unimolecular G4 substrate compared with TMS or TMPyP4. Moreover, the relative ability of Phen-DC3 to inhibit FANCJ helicase activity was strongly dependent on the topology of the G4 substrate. A striking result was that the IC_{50} value for inhibition of FANCJ helicase activity by Phen-DC3 on the unimolecular G4 substrate was 150-fold and 875-fold lower than the IC_{50} values for tetra- and bimolecular G4 substrates. These results were highly reproducible for multiple preparations of FANCJ recombinant protein and DNA substrates. In addition, we tested Phen-DC6, a compound structurally related to Phen-DC3, and the FANCJ helicase inhibition data were similar to the ones obtained for Phen-DC3 (Table 2). Inhibition of FANCJ helicase activity by Phen-DC6 was clearly greatest for the unimolecular G4 substrate compared with the bi- or tetramolecular G4 structures.

We also examined the effect of the G4 ligands on DinG helicase activity on the unimolecular G4 substrate compared with TMS or TMPyP4. Moreover, the relative ability of Phen-DC3 to inhibit FANCJ helicase activity was strongly dependent on the topology of the G4 substrate. A striking result was that the IC_{50} value for inhibition of FANCJ helicase activity by Phen-DC3 on the unimolecular G4 substrate was 150-fold and 875-fold lower than the IC_{50} values for tetra- and bimolecular G4 substrates. These results were highly reproducible for multiple preparations of FANCJ recombinant protein and DNA substrates. In addition, we tested Phen-DC6, a compound structurally related to Phen-DC3, and the FANCJ helicase inhibition data were similar to the ones obtained for Phen-DC3 (Table 2). Inhibition of FANCJ helicase activity by Phen-DC6 was clearly greatest for the unimolecular G4 substrate compared with the bi- or tetramolecular G4 structures.

FIGURE 3. The Fe-S helicase DDX11 fails to unwind unimolecular G4 DNA. DDX11 was tested for helicase activity on various DNA substrates. Helicase reactions (20 μl) were performed at 37 °C for 15 min under standard helicase conditions as described under “Experimental Procedures.” The indicated concentration of DDX11 was incubated with 5′ Poly(A) Zic1 unimolecular G4 DNA substrate (0.25 nm) (panel A), 19-bp forked duplex (0.25 nm) (panel B), TP-G4 tetramolecular DNA substrate (0.25 nm) (panel C), or OX-1 bimolecular G4 DNA substrate (0.25 nm) (panel D). Descriptions of the respective lanes for helicase reactions with the indicated DNA substrates are the same as those described above. Panel E, quantitative analysis of helicase activity on all DNA substrates are shown with S.D. indicated by error bars.

G4 DNA Binding by Phen-DC Compounds as Measured by Fluorescence-induced TO Displacement—To assess if the Phen-DC G4 ligands were able to bind unimolecular G4, we performed FID assays and compared results from experiments with Phen-DC3 or Phen-DC6 with OX-1-G2′, 3′-Poly(A) Zic1-G4, or 5′-Poly(A) Zic1-G4. A schematic principle of the FID assay is shown in Fig. 7A. Initially, fluorimetric titrations with TO and the specified uni- or bimolecular G4 substrates were performed (Fig. 7B). 5′-Poly(A) Zic1-G4 and OX-1-G2′ accommodate the fluorescent probe TO in a very similar way as demonstrated by the similar fluorescence profiles as a function of DNA concentration. The comparison between the two oligonucleotides is thus valid. 3′-Poly(A) Zic1-G4 displayed a lower maximum fluorescence, but the fluorescence profile is similar to that of the other G4 substrates with saturation in the same concentration range (1–2 μM). We next performed G4-FID experiments to evaluate TO displacement induced by ligand binding as measured by fluorescence decrease (Fig. 7C). Phen-DC3 and Phen-DC6 displayed similar activity to displace the TO probe from unimolecular G4 DNA substrates 3′-Poly(A) Zic1-G4 or 5′-Poly(A) Zic1-G4. However, the concentration of Phen-DC6 required to achieve saturation was greater than that of Phen-DC3. Phen-DC3 or Phen-DC6 binding to the bimolecular OX-1-G2′ DNA substrate showed a different behavior compared with the unimolecular G4 substrates with the TO displacement being less efficient. The DC_{50} values corresponding to ligand concentrations required for 50% TO displacement are shown in Fig. 7C.
Deficiency in FANCJ, but Not the Fe-S helicases DDX11 or XPD, Sensitizes Human Cells to the G4 Ligand TMS—The apparent unique ability of FANCJ but not the human DDX11 or XPD helicases to unwind unimolecular G4 DNA in vitro under conditions that all three helicases unwound simple forked duplex substrates raised the question if DDX11 or XPD would confer resistance to a G4 binding ligand. Initially, we tested U2 OS cells for sensitivity to the Phen-DC compounds, but we

**Figure 4.** DinG efficiently unwinds bimolecular and tetramolecular G4 DNA but fails to unwind unimolecular G4 DNA. DinG was tested for helicase activity on various DNA substrates. Helicase reactions (20 μl) were performed at 37 °C for 15 min under standard helicase conditions as described under “Experimental Procedures.” The indicated concentration of DinG was incubated with 5’ Poly(A) Zic1 unimolecular G4 DNA substrate (0.25 nM) (panel A), 19-bp forked duplex (0.25 nM) (panel B), TP-G4 tetramolecular DNA substrate (0.25 nM) (panel C), or OX-1 bimolecular G4 DNA substrate (0.25 nM) (panel D). ssDNA, single-stranded DNA. Descriptions of the respective lanes for helicase reactions with the indicated DNA substrates are the same as those described above. Panel E, quantitative analysis of helicase activity on all the DNA substrates are shown with S.D. indicated by error bars.

**Figure 5.** XPD fails to unwind all three topological G4 DNA substrates. *T. acidophilum* XPD was tested for helicase activity on various DNA substrates. Helicase reactions (20 μl) were performed at 37 °C for 15 min under standard helicase conditions as described under “Experimental Procedures.” The indicated concentration of *T. acidophilum* XPD was incubated with 5’ Poly(A) Zic1 unimolecular G4 DNA substrate (0.25 nM) (Panel A), 19-bp forked duplex (0.25 nM) (Panel B), TP-G4 tetramolecular DNA substrate (0.25 nM) (Panel C), or OX-1 bimolecular G4 DNA substrate (0.25 nM) (Panel D). Descriptions of the respective lanes for helicase reactions with the indicated DNA substrates are the same as those described above. ssDNA, single-stranded DNA.

Deficiency in FANCJ, but Not the Fe-S helicases DDX11 or XPD, Sensitizes Human Cells to the G4 Ligand TMS—The apparent unique ability of FANCJ but not the human DDX11 or XPD helicases to unwind unimolecular G4 DNA in vitro under
found they showed only modest DNA damage induction (data not shown). Therefore, we used the G4 ligand TMS to test for sensitivity of FANCJ-, DDX11-, or XPD-deficient human cells (Fig. 8). Consistent with our previous results (8), FANCJ-depleted U2 OS cells treated with 5 M TMS showed increased γ-H2AX foci compared with siRNA control cells (Fig. 8, B versus C), indicating accumulation of DNA damage. Although DDX11 failed to unwind unimolecular G4 DNA substrates, the Fe-S helicase is able to unwind the bimolecular G2 substrate and, to a lesser extent (at higher protein concentrations), the tetramolecular G4 substrate (this study and Ref. 15). This led us to test human cells depleted of DDX11 by siRNA for TMS sensitivity compared with control siRNA cells. We observed that DDX11-depleted cells were as resistant to TMS as siRNA control cells in the γ-H2AX induction assays; moreover, the level of γ-H2AX was similar to that observed in DMSO-treated cells (Fig. 8, D and G). We also assessed the importance of XPD status for TMS resistance to DNA damage induction by comparing an isogenic pair of human XPD mutant and corrected cell lines. TMS did not increase γH2AX foci in the XPD mutant cell line compared with the control DMSO treatment (Fig. 8, E–G). In control experiments, depletion of DDX11 or FANCJ conferred sensitivity to the DNA cross-linking agent MMC (Fig. 9), consistent with previous findings (22, 30). In addition, the XPD mutant cell line was sensitive to UV irradiation, whereas the corrected XP-D cell line was resistant to UV-induced DNA damage (Fig. 9). Based on these findings, we conclude that cellular deficiency in DDX11 or XPD helicases rendered them sensitive to MMC and UV light, respectively, but not to the G4 ligand TMS.

**DISCUSSION**

Distinct forms of G-quadruplex DNA are known to form in vitro based on their topological architecture. The biological significance of the multiple conformations of G4 DNA is not well understood. A number of proteins are known to interact with G4 DNA (for review, see Refs. 1 and 2); however, their mechanisms of action on specific G4 configurations have not been thoroughly investigated. In this work we focused our studies on the Fe-S cluster DNA helicases and their ability to unwind sin-
gle-stranded or multistranded G-quadruplex DNA structures. The incentive behind this work was provided by biochemical and cellular evidence from several laboratories including ours that the Fe-S helicase FANCJ plays an important role in G4 DNA metabolism by enabling smooth replication of human genomic G-rich DNA sequences predicted to form G-quadruplexes. The potential importance of other sequence-related Fe-S DNA helicases in G4 biology has not largely been addressed; consequently, we examined this issue by evaluating the G4 substrate specificity of several Fe-S DNA helicases including DDX11 and XPD that are genetically linked to diseases characterized by DNA repair and chromosomal instability defects. An emphasis of the current work was to test if the Fe-S helicases could favorably unwind unimolecular G4 DNA, a topological conformation that is entropically favored over multistranded G4 structures.

Our biochemical analysis demonstrates for the first time that FANCJ unwinds unimolecular G4 DNA. Moreover, the ability of FANCJ to unwind unimolecular G4 DNA distinguishes its substrate specificity from the sequence-related Fe-S cluster DNA helicases DDX11, XPD, and DinG, which completely failed to unwind unimolecular G4 under conditions they efficiently unwound a more conventional forked duplex substrate. In fact, the XPD helicase failed to unwind all types of G4 structures, even the bi- and tetramolecular forms. Our findings go against conventional thinking that G4 unwinding is a nonspecific activity of many DNA helicases. Notably, of the helicases reported to be tested for G4 DNA binding and unwinding, only two Fe-S helicases (FANCJ, DDX11) appeared on a recently published list (1), notwithstanding Dna2, which contains an Fe-S cluster in its nuclease domain (32). Two high profile Fe-S DNA helicases with proposed functions in cellular G4 DNA metabolism, human RTXL (33) and C. elegans DOG-1 (7), remain to be biochemically tested for their activity on G4 DNA substrates. Nonetheless, from a biochemical standpoint, our results suggest that FANCJ is indeed a specialized Fe-S helicase in terms of its efficient ability to unwind unimolecular G4 DNA. To our knowledge, the only other DNA helicase demonstrated to biochemically unwind a unimolecular G4 DNA substrate is G4 Resolvase 1 (G4R1) (17); however, the 3’ single-stranded tail required for G4R1 helicase activity on unimolecular G4 DNA (17) is distinct from that of FANCJ, which requires a 5’ single-stranded tail. Importantly, our studies firmly demonstrate that an analysis of G4 DNA unwinding activity by a given helicase must take into account the topological structure of the G4 substrate in addition to other factors (e.g. polarity of single-stranded loading tail).

In the current study we present new evidence characterizing the synthetic G4 ligands Phen-DC3 and Phen-DC6, which potently inhibited FANCJ helicase on the unimolecular G4 substrate compared with a bi- or tetramolecular G4 substrate. This raises the possibility that the Phen-DC compounds and related G4 ligands may be useful tools to probe the molecular specificity of G4 DNA metabolizing enzymes. As demonstrated by other studies, G4 ligands are also useful to investigate G4 metabolism at the cellular level. Here, we showed that TMS induced DNA damage in a manner dependent on FANCJ status. This effect of TMS was unique to FANCJ because the compound did not exert biological effects on human cells deficient in the Fe-S DNA helicases DDX11 or XPD. The potential relevance of the DDX11 in vitro ability to unwind multistranded G4 DNA substrates for its roles in sister chromatid cohesion or DNA repair remains to be determined. Based on our biochemical and cellular results, we suggest that FANCJ is a specialized G4 resolving helicase among the Fe-S cluster helicases and that its ability to unwind unimolecular G4 DNA is likely to be uniquely important for its biological role in DNA replication.

3 S. Kumar Bharti, J. A. Sommers, F. George, J. Kuper, F. Hamon, K. Shin-ya, M.-P. Teulade-Fichou, C. Kisker, and R. M. Brosh, Jr., unpublished data.
Although G4 binding ligands have been applied in the experimental setting in model systems for a number of years, important new advances have arisen quite recently, shedding new light on the existence and consequences of G4 DNA. These studies have built upon a seminal observation that the G4 binding compound TMS induced telomere destabilization by perturbing the interaction of the shelterin single-stranded DNA-binding protein POT1, resulting in apoptosis of cancer cells (31). A synthetic small molecule that was found to stabilize telomeric G-quadruplex DNA in vitro was able to uncap POT1 from telomeric single-stranded DNA in HT1080 cancer cells and trigger a DNA damage response at telomeres (34). Although a number of studies have implicated the importance of G4-resolving helicases in telomere metabolism (35–37), at least some of these helicases are likely to act upon non-telomeric G4-forming DNA sequences as well. A G4 ligand (pyridostatin) promoted growth arrest in human cancer cells by inducing replication- and transcription-dependent DNA damage in genomic regions prone to form G-quadruplexes that are recognized by the human G4-resolving helicase Pif1 (38). Pyridostatin, like other G4 binding ligands, modulated expression of genes with high potential to form G4, including the proto-oncogene SRC (38). The T. acidophilum XPD helicase, an ortholog of human XPD implicated in transcription and nucleotide excision repair, is inactive on G-quadruplex DNA, suggesting that other DNA helicases modulate G4-related events that might occur in the fairly abundant G-rich sequences of the
FANCJ Is a Specialized Fe-S Helicase in G4 DNA Metabolism

genome predicted to form G-quadruplexes. For example, human fibroblasts deficient in the G4-resolving helicases WRN or BLM display up-regulated expression of genes enriched for predicted G4-forming sequences (39). The potential for targeting G-quadruplexes in gene promoters for anti-cancer therapy (40) prompts a greater understanding of the roles of G4 helicases in gene regulation and under what cellular contexts they operate.

In the future it will be of interest to determine if a labeled G4 ligand or antibody directed against G4 DNA with a specific topology co-localizes with FANCJ or other human G4 helicases in addition to PIF1 at specific G4-forming genomic sequences. Experimental studies that address the effect(s) of a helicase mutation/depletion on G4 abundance and G4 ligand-induced DNA damage will be informative. A significant advance in this area would be the direct visualization of accumulated G-quadruplex structures in FANCJ-deficient cells using a G4-specific antibody or molecular beacon attached to a G4-specific DNA binding ligand. As an alternative approach, G4 DNA may be isolated from nuclear extracts of helicase-deficient cells using an affinity approach in which a G4 binding ligand (e.g. N-methyl mesoporphyrin (NMM)) coupled to a Sepharose resin (41) or a biotinylated small molecule derived from N,N′-bis(2-quinolinyl)pyridine-2,6-dicarboxamide (RR82) bound to streptavidin-coated magnetic beads (42) is used to precipitate G4-DNA species. New technologies designed to explore the formation, stability, and function of G-quadruplexes in vivo and how they are affected by proteins such as DNA helicases should yield new insights.

FANCJ-deficient human cells display slow S phase progression (23). The recent demonstration in chicken cells that FANCJ counteracts chromatin compaction by counteracting fork stalling on replication obstacles such as G4 structures (13) begs the question, How coordinated is FANCJ unwinding of G-quadruplexes with the cellular replication machinery, and what factors come into play? There is yet no direct evidence that the 5′ to 3′ FANCJ helicase biochemically interacts with any DNA polymerases; however, FANCJ was shown to interact physically and functionally with the single-stranded DNA binding protein replication protein A (RPA) required for cellular DNA replication (24). The interaction of FANCJ with the BLM helicase (43) may be relevant to replication or repair of a G4-forming sequence as well. Human RTEL has been shown to counteract G4 structure at telomeres (33), but the molecular mechanism is not well understood. Clearly, further studies of FANCJ and other helicases in cellular G4 DNA metabolism are warranted, as evidence suggests a specialization of duties.

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