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Inhibition of BACH1 (FANCI) helicase by backbone discontinuity is overcome by increased motor ATPase or length of loading strand

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ABSTRACT

The BRCA1 associated C-terminal helicase (BACH1) associated with breast cancer has been implicated in double strand break (DSB) repair. More recently, BACH1 (FANCI) has been genetically linked to the chromosomal instability disorder Fanconi Anemia (FA). Understanding the roles of BACH1 in cellular DNA metabolism and how BACH1 dysfunction leads to tumorigenesis requires a comprehensive investigation of its catalytic mechanism and molecular functions in DNA repair. In this study, we have determined that BACH1 helicase contacts with both the translocating and the non-translocating strands of the duplex are critical for its ability to track along the sugar phosphate backbone and unwind dsDNA. An increased motor ATPase of a BACH1 helicase domain variant (M299I) enabled the helicase to unwind the backbone-modified DNA substrate in a more proficient manner. Alternatively, increasing the length of the 5' tail of the DNA substrate allowed BACH1 to overcome the backbone discontinuity, suggesting that BACH1 loading mechanism is critical for its ability to unwind damaged DNA molecules.

INTRODUCTION

The BRCA1 associated C-terminal helicase (BACH1) has been shown to have a role in repair of double strand breaks (DSB) by homologous recombination (HR) (1). BACH1 null cells are hypersensitive to DNA interstrand cross-linking agents (2,3), exhibit diminished BRCA1 foci in untreated cells, and have delayed ionizing radiation (IR) induced BRCA1 foci (4). Interaction between BRCA1 and BACH1 depends on two intact BRCA BRCT repeats and BACH1

phosphorylation (5–7), and this interaction is required for DNA damage induced checkpoint control (8). These findings suggest that BACH1 likely plays a critical role in HR-mediated DSB repair through its interaction with BRCA1.

Genetic importance of BACH1 helicase deficiency in the promotion of breast cancer was suggested by the identification of two females among a cohort of 65 women with early-onset breast cancer who carried two independent germ line sequence changes (P47A or M299I) in the *BACH1* coding region and normal genotypes for *BRCA1* and *BRCA2* (9). The interaction of BACH1 with BRCA1 and the existence of these BACH1 helicase domain missense mutations in a group of early-onset breast cancer patients and not in 200 normal controls have led to the proposal that BACH1 may exert a tumor suppressor function. Importance of BACH1 as a tumor suppressor was demonstrated by the identification of *BACH1* as *Fanci*, the gene defective in Fanconi Anemia (FA) complementation group FA-J (3,10,11). FA is an autosomal recessive disorder characterized by multiple congenital anomalies, progressive bone marrow failure and high risk of cancer [for review, see Ref. (12)]. Cells from FA patients exhibit spontaneous chromosomal instability and hypersensitivity to DNA interstrand cross-linking agents; however, the precise role of BACH1 in the FA pathway remains to be elucidated.

Although genetic data have confirmed the importance of BACH1 in genomic stability, only limited information is available concerning its mechanism of DNA unwinding. BACH1, a member of Helicase Superfamily (SF) 2, was shown to be a 5' to 3' helicase based on its directionality of unwinding a linearized M13 partial duplex substrate (9) and substrate requirement of a 5' ssDNA overhang to unwind a simple oligonucleotide-based duplex substrate (13). BACH1 preferentially binds and unwinds forked duplex DNA substrates and efficiently unwinds a model D-loop structure that is an intermediate of HR (13). In this work, we have determined that BACH1 contacts with both the translocating and the non-translocating strands of the DNA double

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Table 1. Oligonucleotides used in this study

Name	Length (nt)	Sequence (5'→3')
DC26	45	TTTTTTTTTTTTTTTTTCCAAGTAAAACGACGACGGCCAGTGC
TSTEM25	44	GCACTGGCCGTCGTTTTACGGTCGTGACTGGGAAAACCTGGCG
X12-1	50	GACGCTGCCGAATTCGGCTTGCTAGGACATCTTTGCCACGTTGACCCG
X12-1-12-S18-35	47	GACGCTGCCGAATTCGGCTTGCTAGGACATCTTT(SPACER18)CACGTTGACCCG
X12-1-ABASIC	47	GACGCTGCCGAATTCGGCTTGCTAGGACATCTTT(AB-AB-AB)CACGTTGACCCG
X12-1+16T	66	TTTTTTTTTTTTTTTTTGACGCTGCCGAATTCGGCTTGCTAGGACATCTTTGCCACGTTGACCCG
X12-1-16T-51-S18-12	63	TTTTTTTTTTTTTTTTTGACGCTGCCGAATTCGGCTTGCTAGGACATCTTT(SPACER18)CACGTTGACCCG
X12-2	50	CGGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTATGACGTC
X12-2-35-S18-12	47	CGGGTCAACGTG(SPACER18)AAAGATGTCCTAGCAATGTAATCGTCTATGACGTC
X12-2-ABASIC	47	CGGGTCAACGTG(AB-AB-AB)AAAGATGTCCTAGCAATGTAATCGTCTATGACGTC
X12-2-31	31	CGGGTCAACGTGGGCAAAGATGTCCTAGCAA
X12-2-12-S18-16	28	CGGGTCAACGTG(SPACER18)AAAGATGTCCTAGCAA

helix are essential for BACH1 to track along the sugar phosphate backbone and unwind dsDNA. Helicase inhibition by a backbone modification can be alleviated by either enhancing BACH1 ATPase activity or lengthening the 5' ssDNA tail of the DNA substrate that BACH1 uses for loading.

MATERIALS AND METHODS

Proteins

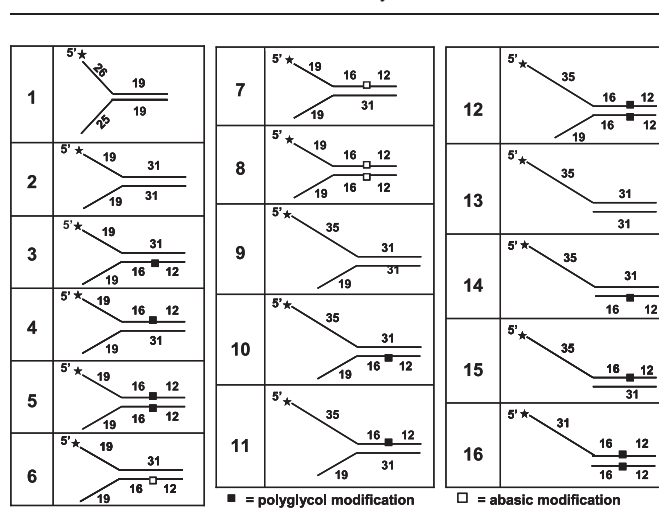
Baculovirus encoding BACH1-WT, BACH1-P47A, BACH1-M299I, or BACH1-K52R with a C-terminal FLAG tag was used to infect High Five insect cells and the recombinant BACH1 protein was purified as previously described (9). At least three independent protein purifications of the purified wild-type and mutant recombinant BACH1 proteins were performed, yielding very similar results in catalytic ATPase and helicase assays, indicating uniformity in the determined specific activities of these proteins.

DNA substrates

PAGE-purified oligonucleotides used for the preparation of DNA substrates were purchased from either Loftstrand Labs (Gaithersburg, MD) or Midland Certified Reagent Co. (Midland, TX) and are listed in Table 1. DNA duplex substrates were 5'-³²P-end labeled and prepared as described previously (14). DNA substrates are shown in Table 2.

Radiometric strand displacement helicase assays

Helicase assay reaction mixtures (20 μl) contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 2% glycerol, 100 ng/μl BSA, 2 mM ATP, 10 fmol of the specified duplex DNA substrate (0.5 nM DNA substrate concentration), and the indicated concentrations of BACH1 helicase. Helicase reactions were initiated by the addition of BACH1 and then incubated at 30°C for 15 min unless otherwise indicated. Reactions were quenched in the presence of a 10-fold excess of unlabeled oligonucleotide with the same sequence as the labeled strand to prevent reannealing and products resolved on non-denaturing 12% (19:1 acrylamide: bisacrylamide) polyacrylamide gels and quantitated as previously described (13).

Table 2. DNA substrates used in this study

Helicase sequestration experiments

For helicase sequestration studies, BACH1 (4.8 nM) was preincubated with the indicated concentrations (0–25 nM) of the unlabeled forked duplex DNA molecule (competitor) in standard helicase reaction buffer as described above along with 2 mM ATP in a total reaction mixture volume of 20 μl for 3 min at 30°C. The reactions were initiated with 10 fmol of radiolabeled forked duplex molecules (Substrate 1) and incubated for 15 min at 30°C. Reactions were quenched and resolved on native polyacrylamide gels. Helicase data (% control) is expressed relative to the control reactions lacking the competitor DNA.

DNA binding assays

Protein–DNA binding mixtures (20 μl) contained the indicated concentrations of BACH1 and 0.5 nM of the specified ³²P-end-labeled DNA substrate in the same reaction buffer as that used for helicase assays (see above) containing 2 mM ATPγS. The binding mixtures were incubated at 24°C for 30 min after the addition of BACH1 and analyzed as previously described (13).

ATPase assays

For k_{cat} determinations, ATPase assay reaction mixtures (25 μl) contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM MgCl_2 , 2 mM dithiothreitol, 2% glycerol, 100 ng/ μl BSA, 82 nM forked duplex or single-stranded 45mer DNA effector concentration, 0.8 mM [^3H]ATP, and 77 nM BACH-WT or 77 nM BACH1-M299I. Reactions were initiated by the addition of BACH1 and incubated at 30°C. Aliquots (5 μl) were removed at 15 min intervals (0–45 min) and evaluated by thin layer chromatography as described previously (15). Less than 20% of the substrate ATP was consumed in the reaction over the entire time course (45 min) of the experiments. For K_{eff} determinations, ATPase reaction mixtures (20 μl) contained the same concentrations of components as described above except forked duplex DNA effector concentrations ranged from 0 to 75 nM and the concentration of BACH1-WT or BACH1-M299I was 50 nM. K_{eff} values were determined using linear regression analysis from double reciprocal plots of ATP (pmol) hydrolyzed versus forked-duplex DNA effector concentration.

Fluorometric dye displacement helicase kinetic assays

Fluorescence stopped-flow kinetic experiments were performed using an Applied Photophysics SX.18MV stopped-flow reaction analyzer. Hoechst 33258 dye interacted into the dsDNA substrate was excited at a wavelength of 344 nm, and the fluorescence emission was monitored at wavelengths >400 nm with a 400 nm long pass filter from Edmund Optics (Barrington, NJ). Experiments were carried out in the two-syringe mode where BACH1 and ATP were preincubated at 30°C in one syringe for 1 min, while the DNA substrate was preincubated with Hoechst 33258 dye (100 nM, Molecular Probes) at 30°C in the second syringe. Each syringe contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM MgCl_2 , 2 mM dithiothreitol, 2% glycerol, and 100 ng/ μl BSA. Final concentrations of BACH1 (4.8 nM), ATP (2 mM), Hoechst 33258 dye (100 nM) and DNA fork substrate (0.8 nM) in the syringe were double that of the final concentration in the reaction. Equal volumes (60 μl) of sample from both syringes were mixed to initiate the reaction, which took place at 30°C. One thousand data points were collected from monitoring 20 μl of each kinetic time course reaction. For converting the output data from volts to percent fluorescence decrease, a time course with the same set up was performed except with only the unannealed oligonucleotides instead of the forked duplex substrate. Data were then normalized by defining the voltage obtained with the unannealed oligonucleotide as 100% fluorescence decrease. In control reactions, no fluorescence change was detected in reaction mixtures containing BACH1-WT helicase and DNA substrate with intercalated Hoechst 33258 dye but lacking ATP. In addition, no fluorescence change was observed when the BACH1-K52R ATPase-helicase dead mutant protein was incubated with ATP and the DNA substrate, indicating that the signal detected was a consequence of BACH1 helicase activity that is dependent on ATP hydrolysis.

RESULTS

BACH1 polymorphisms differentially impact catalytic activities

To evaluate the biochemical effects of two helicase domain BACH1 missense polymorphisms (P47A, M299I) on enzymatic function, we compared their unwinding activities with wild-type BACH1 on a preferred substrate, a forked duplex DNA structure (Table 2, Substrate 1). BACH1-P47A weakly unwound the forked duplex in an ATP- and protein concentration-dependent manner (Figure 1A, lanes 2 and 3; Figure 1B), whereas BACH1-K52R that has an amino acid substitution of the invariant lysine in motif I completely failed to unwind the forked duplex substrate (Figure 1B). In stark contrast, BACH1-M299I efficiently unwound the DNA substrate (Figure 1A, lanes 4 and 5, Figure 1B). Quantitative analyses demonstrated that BACH1-M299I helicase activity was ~1.8-fold greater than BACH1-WT at an enzyme concentration of 0.3 nM (Figure 1B). At least three independent protein purifications of the purified wild-type and mutant recombinant BACH1 proteins were performed, and very similar results in the helicase measurements were obtained, indicating that the differences in helicase activity observed were reproducible.

Preferential unwinding of the forked duplex by BACH1 reflects its ability to bind the DNA substrate more efficiently compared to a simple 5' tailed duplex (13). Gel-shift assays demonstrated that a greater fraction of the forked duplex is bound by BACH1-M299I compared to BACH1-WT throughout the protein concentration range used for the helicase assays (Supplementary Figure 1). The maximal difference in DNA binding between the mutant and wild-type proteins was observed at the highest protein concentration tested, 4.8 nM, in which ~40% of the forked duplex was bound by BACH1-M299I compared to 15% substrate bound by BACH1-WT. BACH1-P47A, however, displayed similar binding compared to BACH1-WT (data not shown). The wild-type and variant enzymes were tested for DNA-dependent ATPase activity using the forked duplex as the DNA effector at a concentration of ATP (0.8 mM) in which <20% of the nucleotide was hydrolyzed during the entire time course of the reaction. Both BACH1-WT and BACH1-M299I displayed a similar dependence on the forked duplex for ATPase activity as evidenced by their K_{eff} values in the same range (Table 3); however, the turnover rate constant (k_{cat}) was 6.7-fold greater for BACH1-M299I, indicating that the variant hydrolyzed ATP substantially better than the wild-type enzyme (Table 3). BACH1-P47A catalyzed ATP hydrolysis poorly as evidenced by its low k_{cat} , 12-fold less than BACH1-WT (Table 3). Similar results from ATPase assays for the wild-type and mutant (P47A, M299I) BACH1 proteins were obtained at an ATP concentration of 2 mM and with several different preparations of the mutant and wild-type BACH1 proteins (data not shown). The k_{cat} values for ATP hydrolysis using the single-stranded 45mer oligonucleotide DC26 (Table 1) were 28.4 ± 4.8 per minute and 218 ± 2.1 per minute for BACH1-WT and BACH1-M299I, respectively, indicating that the differences in ATPase activity between BACH1-WT and BACH1-M299I proteins were observed for both forked duplex and ssDNA molecules. These results suggest that the increased helicase activity of BACH1-M299I relative to BACH1-WT

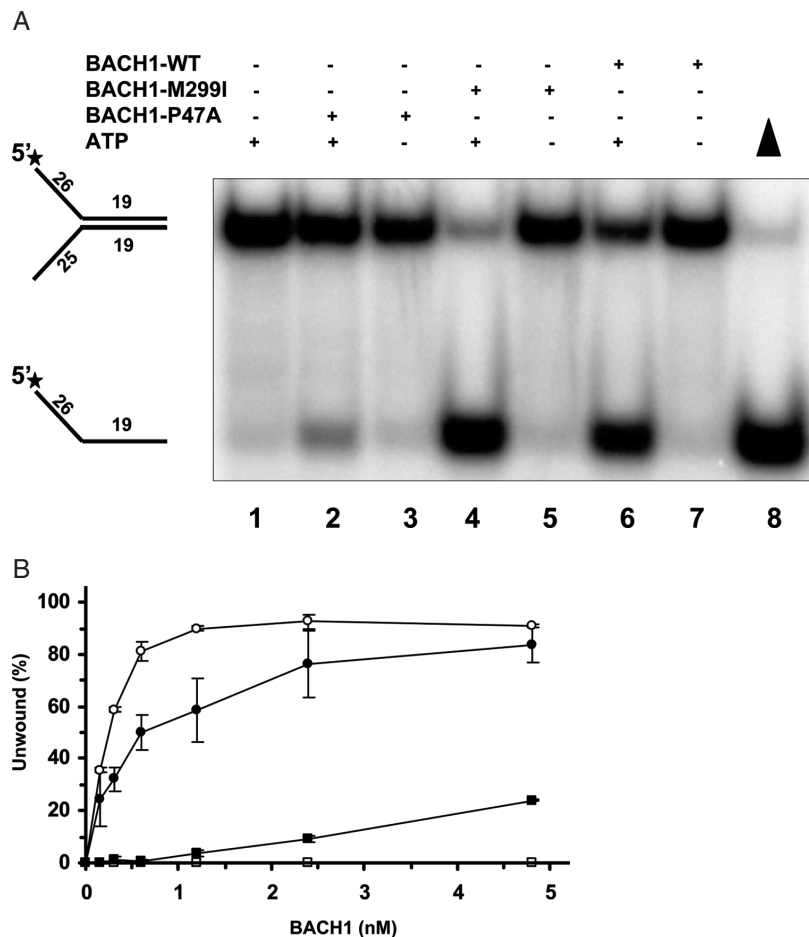


Figure 1. Differential effects of BACH1 polymorphisms on helicase function. Helicase reactions (20 μ l) were performed by incubating 4.8 nM BACH1-WT or BACH1 variant (BACH1-M299I, BACH1-P47A) as indicated with 0.5 nM forked duplex DNA substrate (Substrate 1) at 30°C for 15 min in the presence or absence of ATP (2 mM) under standard helicase assay conditions as described under 'Materials and Methods.' (A) Lane 1, no enzyme control; Lanes 2–7, indicated BACH1 protein in the presence or absence of ATP; Lane 8, heat-denatured DNA substrate control. A phosphorimage of a typical gel is shown. (B) Quantitative helicase data represent the mean of at least three independent experiments with standard deviation (SD) indicated by error bars, as is the case for the remaining figures. Filled circles, BACH1-WT; open circles, BACH1-M299I; filled squares, BACH1-P47A; open squares, BACH1-K52R.

Table 3. ATP hydrolysis by BACH1-WT and BACH1 variants using forked duplex as the DNA effector

Protein	K_{eff} (nM) \pm SD	k_{cat} (min^{-1}) \pm SD
BACH1-WT	2.5 \pm 2	25.4 \pm 3.0
BACH1-M299I	4.5 \pm 0.7	167 \pm 15.9
BACH1-P47A	—	2.11 \pm 2.7

ATP hydrolysis reactions were as described under 'Materials and Methods' using 77 nM BACH1-WT or BACH1-M299I and 82 nM of the forked duplex effector (Table 2, DNA molecule 1) for the k_{cat} determination. For K_{eff} determination, 50 nM BACH1-WT or BACH1-M299I was used with the forked duplex DNA effector concentrations ranging from 0 to 75 nM.

is accompanied by better binding to the DNA substrate and significantly improved efficiency for ATP hydrolysis in the presence of the forked duplex. Although BACH1-M299I unwound the forked duplex better than BACH1-WT, the difference was not nearly as great as the observed effects on ATP hydrolysis. BACH1-P47A is severely compromised in both its ATPase and helicase activity; however, a low residual ATP-dependent unwinding activity exists.

BACH1 helicase activity on forked duplex substrates with backbone modifications or abasic sites within the duplex region

Since little is known about the BACH1 helicase mechanism, we investigated the importance of structural and chemical features of the DNA duplex for unwinding. The forked duplex tested for BACH1 helicase activity contained either a polyglycol modification to the sugar phosphate backbone or a set of three adjacent abasic sites positioned 16 bp from the fork entry site on either the translocating (top) or non-translocating (bottom) strand that the enzyme is presumed to translocate based on its demonstrated 5' to 3' directionality of unwinding (9,13). These two types of structural perturbations to the DNA double helix enabled us to assess the relative importance of backbone integrity or base contacts for BACH1 as it tracks through the duplex and catalytically separates the two strands.

BACH1-WT was profoundly sensitive to the polyglycol backbone modification in either the translocating or non-translocating strands of the duplex. No unwinding of the

backbone modified DNA substrates by BACH1-WT (up to 20 nM) could be detected in the 15 min reaction (Figure 2) or 1 h incubation (data not shown). Thus BACH1-WT helicase activity does not display strand-specific inhibition with respect to the strand that it translocates on as observed for other helicases [vaccina virus NPH-II (16), *Escherichia coli* Dda, bacteriophage (17,18), T7 gene 4 (19)], suggesting that BACH1 has a unique ability to sense both strands of the DNA duplex during the unwinding reaction. Indeed, we found that unwinding of the same forked duplex substrates by human RECQ1, a 3' to 5' helicase, is inhibited in a strand-specific manner such that the polyglycol modification in only the translocating (bottom) strand inhibited unwinding (manuscript in preparation). It should be pointed out, however, that studies of *E.coli* DnaB (SF4) (20) and hepatitis C virus NS3 (SF2) (21) helicases have suggested that transient interactions of the helicase with the displaced strand are important as well.

We next examined the ability of BACH1-M299I to unwind the backbone modified DNA substrates. For the non-translocating strand modification, BACH1-M299I unwinding was reduced compared to the helicase activity on the control (unmodified) substrate throughout the protein titration range; however, the M299I mutant retained the ability to unwind the DNA substrate with the non-translocating strand modification in a protein concentration dependent manner, exhibiting up to 40% substrate unwound at 19 nM M299I helicase (Figure 2). The translocating strand modification in the backbone further inhibited BACH1-M299I helicase; only 5% of the backbone modified substrate was unwound at 2.5 nM helicase concentration compared to ~50% of the control substrate. At the highest helicase concentration tested (19 nM), BACH1-M299I unwound ~10% of the DNA substrate with the backbone modification in the translocating strand. Nearly complete inhibition was observed for the substrate with backbone modifications in both strands for either BACH1-WT or BACH1-M299I at all protein concentrations tested (data not shown). These results indicate that both the BACH1-WT and BACH1-M299I proteins are sensitive to backbone modifications in either the non-translocating or translocating strands of the DNA substrate; however, the M299I mutant, unlike BACH1-WT, retains the ability to unwind the backbone modified DNA substrates to different extents, depending on which strand the modification is placed, throughout the helicase titration range.

We also tested the ability of BACH1 to unwind a DNA substrate containing three adjacent abasic sites in either the top or bottom strand of the duplex at the same position as the polyglycol linkage. BACH1-WT retained the ability to unwind the forked duplex substrate with the abasic sites in the non-translocating strand similar to the control substrate (Figure 3). The presence of the abasic sites in the translocating strand of the DNA substrate resulted in a 1.6-fold reduction of BACH1-WT helicase activity. The presence of abasic sites in both strands resulted in almost complete inhibition of BACH1-WT helicase activity. Thus BACH1-WT is significantly more sensitive to a perturbation of sugar phosphate backbone continuity than the absence of bases in the duplex region, suggesting that electrostatic interactions of BACH1 helicase with the sugar phosphate backbone are more important than base-stacking interactions. In contrast to wild-type

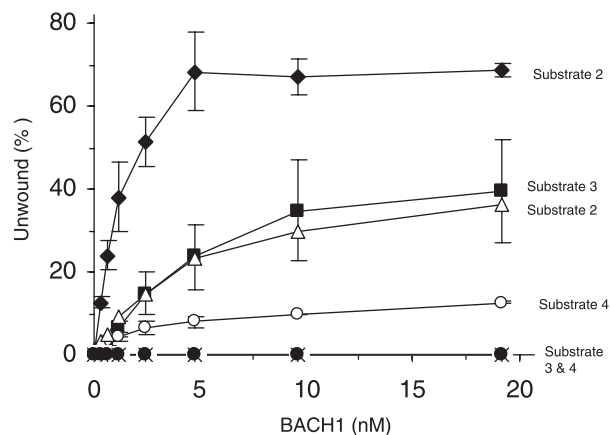


Figure 2. Effect of polyglycol backbone modifications on BACH1 helicase activity. The indicated concentrations of BACH1-WT or BACH1-M299I were incubated with 0.5 nM DNA substrate containing the polyglycol modification (Substrates 2–4) at 30°C for 15 min under standard helicase assay conditions as described under ‘Materials and Methods.’ Quantitative analyses of BACH1 helicase data are shown. Open triangle, BACH1-WT, Substrate 2; filled circle, BACH1-WT, Substrate 3; cross, BACH1-WT, Substrate 4; filled diamond, BACH1-M299I, Substrate 2; filled square, BACH1-M299I, Substrate 3; open circle, BACH1-M299I, Substrate 4.

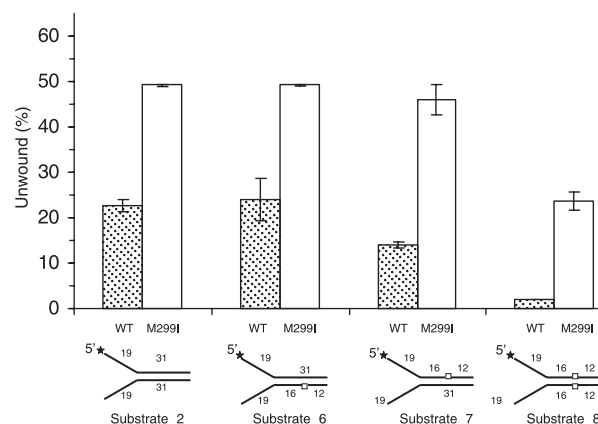
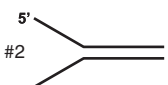
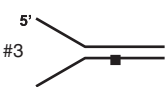

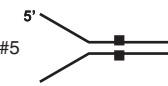


Figure 3. Effect of abasic sites on BACH1 helicase activity. 4.8 nM BACH1-WT or 2.4 nM BACH1-M299I was incubated with 0.5 nM DNA substrate containing the three adjacent abasic sites (Panel B, Substrates 2,6,7,8) at >30°C for 15 min under standard helicase assay conditions as described under ‘Materials and Methods.’ Quantitative analyses of BACH1 helicase data are shown.

BACH1, the M299I variant was not inhibited to any appreciable extent by the presence of abasic sites in the non-translocating or translocating strands of the DNA substrate. However, the presence of abasic sites in both strands reduced BACH1-M299I helicase activity 2-fold compared to the control substrate. Collectively, BACH1-M299I has a greater tolerance for abasic sites or backbone modifications nested within the duplex in either the translocating or non-translocating strands of the DNA substrate compared to BACH1-WT.

The complete failure of wild-type BACH1 enzyme to unwind the forked duplex substrates with the backbone modification in either strand of the duplex raised the possibility that the polyglycol moiety perturbed the ability of the protein to interact with or bind the DNA substrate. To address this, BACH1-WT was tested for DNA-dependent ATPase activity

Table 4. ATP hydrolysis by BACH1-WT or M299I using forked duplex DNA effectors with backbone polyglycol modifications

DNA Effector	k_{cat} (min^{-1}) \pm SD BACH1-WT	BACH1-M299I
#2 	28.0 \pm 1.2	183 \pm 8.8
#3 	31.0 \pm 2.4	157 \pm 4.5
#4 	27.4 \pm 1.4	162 \pm 13
#5 	27.0 \pm 0.8	162 \pm 26

ATP hydrolysis reactions were as described under 'Materials and Methods' using 77 nM BACH1-WT or BACH1-M299I, and 82 nM (forked-duplex molecules) of the indicated DNA effector.

using the unmodified or backbone modified DNA molecules as effectors. BACH1-WT hydrolyzed ATP with a similar efficiency for each of the DNA effectors (Table 4). BACH1-M299I was also found to hydrolyze ATP similarly and significantly better than the wild-type enzyme (Table 4). A quantitative comparison of the k_{cat} values for ATP hydrolysis using the various modified or unmodified DNA effects demonstrated a difference in specificity ATPase activity of 5- to 6.5-fold. BACH1-WT and BACH1-M299I were found to bind the modified duplex molecules (data not shown), consistent with the fact that BACH1 is a DNA-dependent ATPase (9).

BACH1 partially unwinds the forked duplex but becomes sequestered by the backbone modification

Certain helicases have been found to be sequestered by DNA molecules harboring various types of covalent lesions (22). Pre-incubation of BACH1 (4.8 nM) with increasing concentrations of unlabeled forked duplex molecules inhibited unwinding of the radiolabeled tracker DNA substrate that was added subsequently to the reaction mixtures. Pre-incubation of BACH1 (4.8 nM) with forked duplexes containing the backbone modification in either the translocating or non-translocating strands inhibited BACH1 unwinding of the tracker DNA substrate to a significantly greater extent than the unmodified forked duplex (Figure 4). These results suggest that BACH1 was preferentially sequestered by the backbone modification irrespective of strand status.

Since the strand displacement assay conventionally employed to monitor helicase activity does not measure partial unwinding, we employed a dye displacement assay (Figure 5A) to address the hypothesis that BACH1-WT unwound the duplex (16 bp) adjacent to the fork and became trapped at the vicinity of the backbone modification, leaving the remaining duplex (12 bp) intact. Incubation of BACH1-WT (4.6 nM final concentration) with the unmodified forked duplex (0.8 nM final concentration) in the presence of ATP

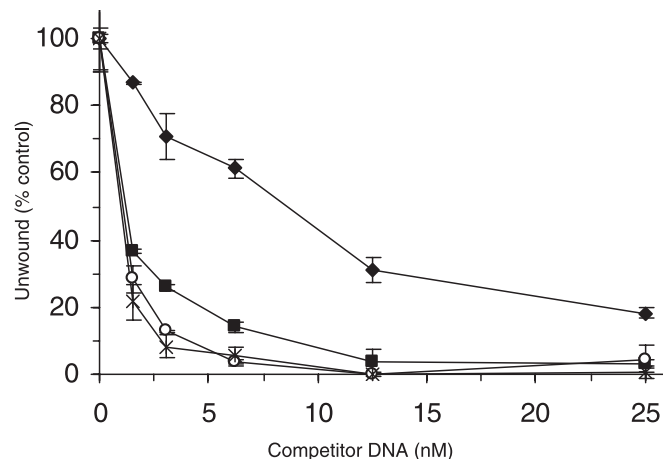


Figure 4. BACH1-WT is preferentially sequestered by DNA molecules with backbone polyglycol modifications in the duplex region. Sequestration assays with 4.8 nM BACH1-WT and increasing concentrations (0–25 nM) of the indicated forked duplex competitor DNA molecules were performed as described under 'Materials and Methods.' Quantitative analyses of the helicase data are shown. Filled diamonds, unmodified forked duplex; filled squares, forked duplex with backbone modification in top (translocating) strand; open circles, forked duplex with backbone modification in bottom (non-translocating) strand; cross, duplex with modification in both strands.

(2 mM) under multiple turnover conditions resulted in a time-dependent fluorescence decrease during the 200 sec interval (Figure 5B). In the absence of ATP, no fluorescence change was detected (data not shown). In addition, no fluorescence change was observed when the BACH1-K52R ATPase dead mutant protein was incubated with ATP and the DNA substrate (data not shown), indicating that the signal detected was a consequence of BACH1 helicase activity that is dependent on ATP hydrolysis. As observed for the unmodified substrate, incubation of BACH1-WT with ATP and the DNA substrate containing a backbone modification in either the translocating or non-translocating strands resulted in a fluorescence change that was dependent on time (Figure 5B), indicating that the fluorescent dye is displaced due to BACH1 helicase activity. However, the percent fluorescence decrease for the assays with the modified substrates was less than that observed for the unmodified forked duplex, indicating that BACH1 partially unwinds the duplex region between the fork entry site and the backbone modification, and becomes trapped by the backbone perturbation.

Inhibition of BACH1 helicase activity by the polyglycol backbone modification is overcome by increasing the length of the 5' ssDNA tail

Previously we reported that BACH1 helicase activity on 5' tailed or forked duplex substrates increased proportionately as the 5' ssDNA tail was increased from 15 to 35 nt (13), raising the possibility that features of the DNA substrate that influence enzyme loading may affect the unwinding event. This prompted us to study the effect of the backbone modifications on BACH1 unwinding of DNA substrates in which the 5' tail was increased to the optimal 35 nt compared to the 19 nt 5' tail in the original set of substrates. As shown

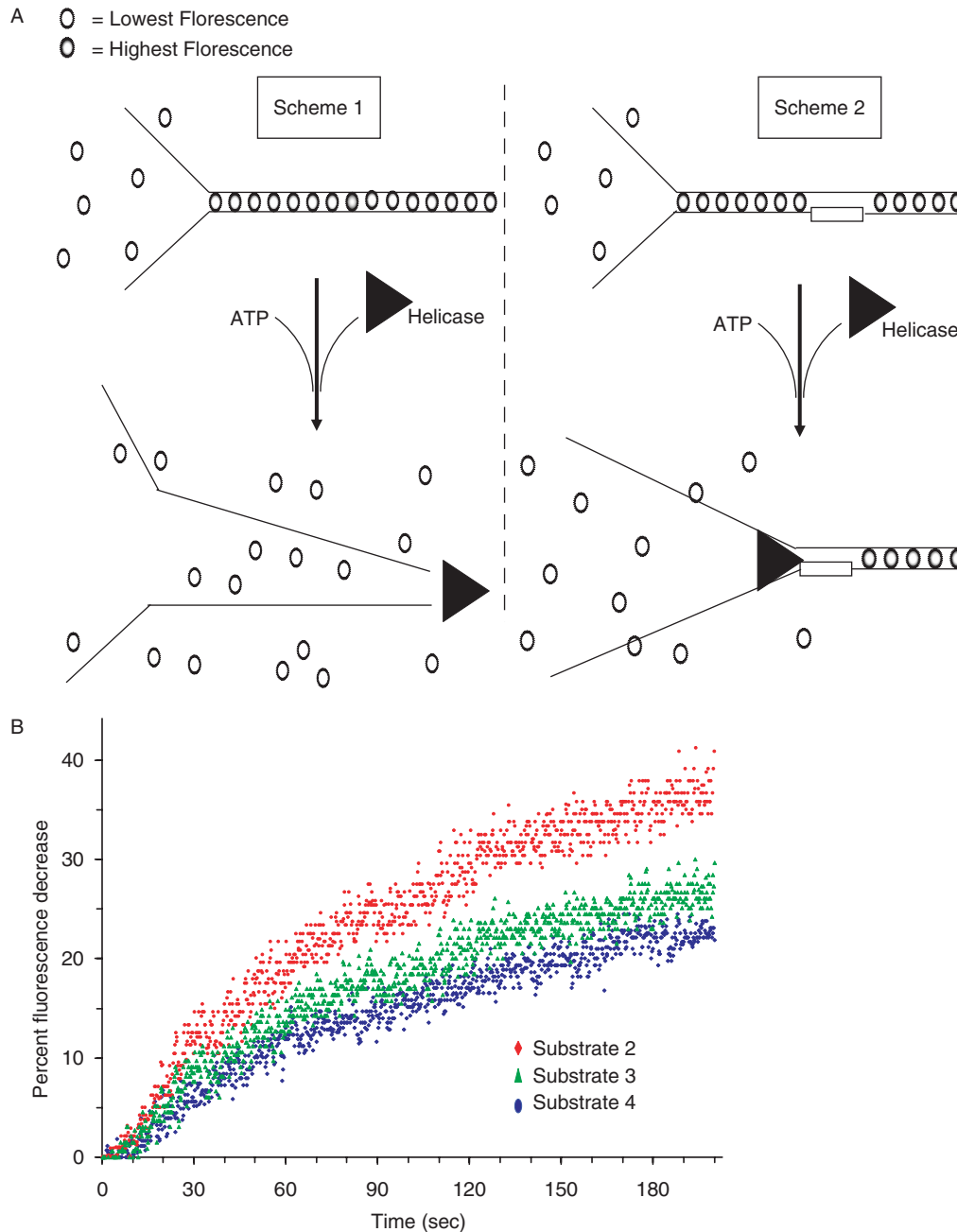


Figure 5. Partial unwinding of backbone modified DNA substrates by BACH1-WT helicase. (A) Schematic of the dye displacement assay to measure helicase activity. The dye molecules are pre-bound to the duplex DNA substrate (dark ovals), causing them to fluoresce. As the dsDNA is unwound, the dye molecules are displaced (open ovals) which is measured by a decrease in the amount of fluorescence. In Scheme 1, the unmodified DNA substrate is fully unwound by the helicase, resulting in a maximal change in fluorescence. In Scheme 2, the backbone modified DNA substrate is partially unwound by the helicase, resulting in a smaller change in fluorescence since a population of dye molecules remain intercalated within the residual duplex. (B) Kinetics of BACH1-WT helicase activity on the unmodified or specified polyglycol modified DNA substrate as measured by the dye-displacement assay. 0.8 nM DNA substrate pre-incubated with 100 nM Hoechst dye was rapidly mixed with 4.6 nM BACH1-WT and 2 mM ATP. Unwinding of the DNA substrate, as shown by percent fluorescence decrease, was measured as a function of time. Red, unmodified Substrate 2; green, Substrate 3 with bottom strand backbone modification; blue, Substrate 4 with top strand backbone modification.

in Figure 6A, backbone modifications to the top or bottom strand of the forked duplex with a 35 nt 5' tail did not have a significant effect on BACH1 helicase activity compared to the control substrate. Polyglycol modifications to both strands reduced BACH1 helicase activity to ~ 45% that of the control. Similar observations for this series of backbone modified substrates were observed at lower BACH1 protein

concentrations in which the percentage of control DNA substrate unwound was comparable to that observed for the forked duplex with a 19 nt 5' tail (data not shown). For the DNA substrates with only a 35 nt 5' tail (Figure 6B), BACH1 helicase activity was also unaffected by the non-translocating strand modification, and only reduced by 20% that of the control for the substrate with the translocating

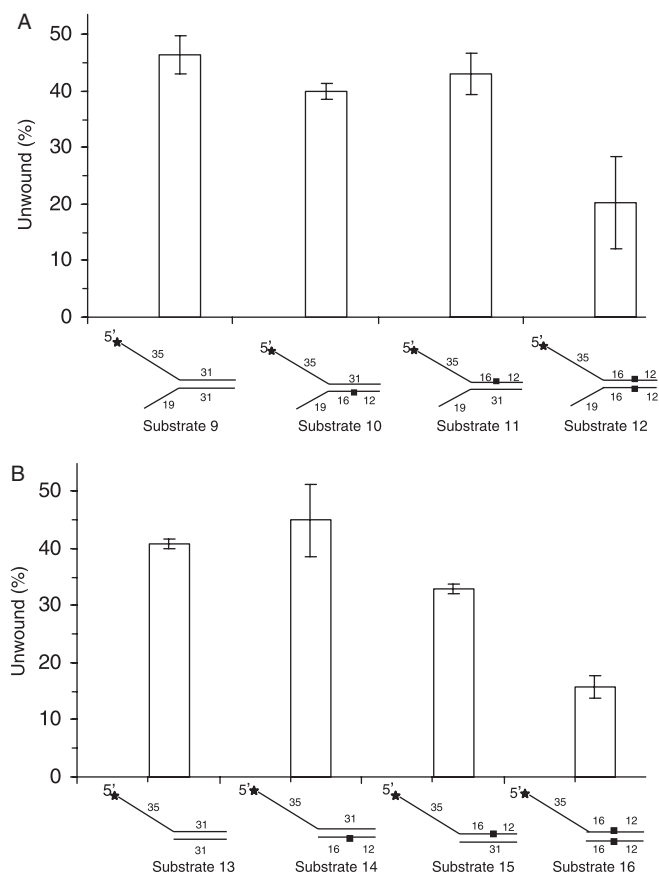


Figure 6. Inhibition of BACH1 helicase activity by the polyglycol backbone modification is overcome by an increased length in the 5' ssDNA tail. 4.8 nM BACH1-WT was incubated with the indicated polyglycol modified forked (35 nt 5' tail, 19 nt 3' tail) duplex [(A) Substrates 9–12] or simple overhang (35 nt 5' tail) duplex [(B) Substrates 13–16] at 30°C for 15 min under standard helicase assay conditions as described under 'Materials and Methods'. Quantitative analyses of BACH1 helicase data are shown from at least three independent experiments with SD indicated by error bars.

strand modification. Backbone modifications in both strands inhibited BACH1 helicase activity on the 35 nt 5' tailed substrate to a level ~40% that of the control. These results indicate that BACH1 helicase inhibition by the polyglycol backbone modification in either strand of the duplex can be successfully overcome by increasing the length of the 5' ssDNA tail the enzyme used for loading on the partially duplex substrate.

DISCUSSION

The molecular and genetic functions of the BACH1 helicase in DNA repair and the molecular pathology of FA due to helicase dysfunction remain to be fully characterized. In an effort to better understand the biochemical properties of BACH1, we have investigated the catalytic activities of BACH1 and two associated polymorphic variants. We have determined that BACH1 helicase interactions with the sugar phosphate backbone of both strands of the duplex are important as BACH1 tracks along the DNA molecule and separates the strands. The backbone tracking mechanism of BACH1 is distinct from that of NPH-II and other SF2 helicases

suggesting that BACH1 has a specialized unwinding mechanism. Very recently, the hepatitis C virus NS3 RNA helicase (SF2) was found to readily unwind RNA duplexes that contained long stretches of polyglycol linkages in either the translocating or non-translocating strands (23). The authors suggested that the large kinetic step size (18 bp) (24) may enable the RNA helicase to 'step over' the large polyglycol lesions in its track. Measurements of the physical and kinetic step size of BACH1 helicase will be useful to understanding how this DNA helicase is completely blocked from unwinding the duplex DNA substrates with a single 18 atom polyglycol linker in either the translocating or non-translocating strands.

Previously, we reported that BACH1 helicase activity is inhibited by a polyglycol modification positioned adjacent to the duplex region in the 5' ssDNA tail, but not the 3' ssDNA tail, of a forked duplex substrate (13). This result is in contrast to our findings in this study showing that BACH1 was sensitive to disruption of backbone continuity in either strand of the DNA duplex region. Thus, the effects of backbone modifications on BACH1 helicase activity may be dependent on whether the enzyme is in the initiation or elongation phases of the unwinding reaction.

Interruption of BACH1 helicase activity in the vicinity of the backbone modification on either the translocating or non-translocating strand leaves the remaining duplex (~12 bp) intact. Although there have been reports of spontaneous melting of the final 9–11 bp of duplex DNA substrates partially unwound by other helicases (25–27), this was not observed in the BACH1 helicase reactions with the backbone modified DNA substrates as evidenced by the complete lack of strand displacement measured by the radiometric helicase assay. Spontaneous fraying of the partially unwound DNA substrate acted upon by BACH1 might be expected based on the theoretical thermal stability (T_m) of the 12 bp duplex calculated from G/C content and salt concentration which is 2°C below the reaction temperature of 30°C. It is conceivable that the sequestration of BACH1 by the polyglycol obstacle or its preferential ability to bind forked DNA structures (13) may contribute to stabilization of the remaining duplex. Unlike a number of human RecQ helicases which have been shown to catalyze strand annealing of complementary ssDNA molecules (28–32), strand annealing by BACH1 is very weak (data not shown), suggesting that this activity does not contribute to the lack of complete unwinding of the polyglycol substrates by BACH1.

BACH1 partially unwound the backbone modified DNA substrates and became sequestered, indicating that BACH1 was not able to effectively unwind past the obstacle. However, by increasing the length of the 5' ssDNA tail used for helicase loading, BACH1 was able to efficiently unwind the backbone-modified DNA substrates. This finding should be considered in light of recent studies which also indicate the importance of DNA substrate loading elements for helicase function. Increasing the length of the ssDNA overhang was shown to enhance Dda catalyzed duplex DNA unwinding (33). Similarly, multiple NS3 helicase molecules bound to the ssDNA loading region of a partial duplex substrate are required for optimal unwinding (34). In addition to DNA unwinding, multiple helicase molecules loaded on the same ssDNA molecule may cooperate to facilitate protein displacement (35), an anticipated function of certain

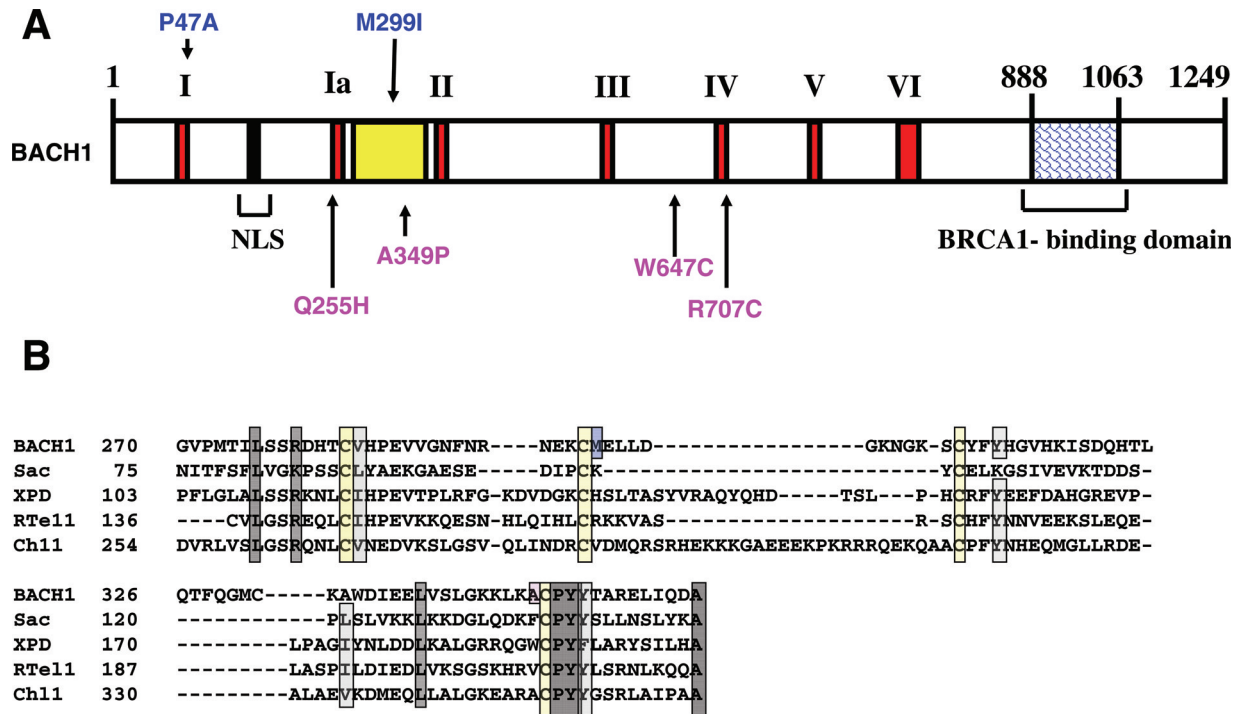


Figure 7. BACH1 protein domains and missense mutations. (A) Cartoon depicting the BACH1 protein with the conserved helicase motifs indicated by red boxes and the positions of the metal binding domain (yellow), nuclear localization sequence (NLS), and BRCA1 interaction domain. BACH1 polymorphic variants (P47A, M299I) are indicated above the protein schematic in blue. Missense mutations (Q255H, A349P, W647C, R707C) genetically linked to Fanconi Anemia (Complementation Group J) are indicated below the protein schematic in pink. (B) Sequence alignment of the region between the Walker A (motif I) and Walker B (motif II) boxes that includes the Fe-S domain for *Sulfolobus acidocaldarius* XPD (Sac) and a number of human DNA helicases (BACH1 (FANCI), XPD, RTe11 and Ch11) as adapted from Ref. (39). Conserved cysteine residues of the metal binding domain are highlighted in yellow. The position of a BACH1 amino acid substitution (M299I) adjacent to a conserved cysteine residue and representing a BACH1 polymorphic variant is highlighted in blue. An amino acid substitution (A349P) arising from a mutation genetically linked to Fanconi Anemia (Complementation Group J) and adjacent to a conserved cysteine residue is highlighted in pink.

helicases. It was proposed that the presence of multiple motors may serve to prevent backward displacement on the ssDNA, resulting in an elevated force production to displace protein from the DNA molecule (35). For BACH1, the length of the 5' ssDNA loading tail used by the helicase during initiation has dramatic consequences for its ability to unwind past the backbone obstacle within the duplex. This may be a consequence of the loading of numerous functionally active BACH1 helicases on the DNA substrate with a longer 5' ssDNA tail under multiple turnover conditions. Attempts to perform single turnover BACH1 helicase experiments were inconclusive since very little unwinding of the radiolabeled substrates pre-incubated with BACH1 was detected when an excess of cold competitor DNA molecule was added with ATP to initiate the reaction (data not shown). The observation that BACH1 partially unwinds the backbone modified forked duplex with a shorter 19 nt 5' ssDNA tail and is sequestered by the backbone perturbation raises the possibility that wild-type BACH1 enzyme may be pushed forward by other BACH1 helicase molecules loaded behind it to complete unwinding.

The two BACH1 polymorphisms (P47A, M299I) exerted dramatically different effects on the catalytic activities of the respective proteins. Although the P47A mutant was found to be completely devoid of helicase activity on an M13 partial duplex substrate (9), we detected a low residual unwinding of the preferred forked duplex substrate and a

small but detectable DNA-dependent ATPase activity. BACH1-M299I helicase, on the other hand, displayed a vastly greater DNA-dependent ATPase activity compared to BACH1-WT; however, its ability to unwind unadducted forked duplexes was only improved by <2-fold under multiple turn-over conditions. BACH1-M299I also bound the forked duplex better than wild-type BACH1, suggesting that the amino acid replacement may stabilize the enzyme: DNA interaction to some extent.

Interestingly, the position of the M299I polymorphism is between the conserved helicase motifs Ia and II, a region found to be involved in ssDNA binding for the SF1 Rep helicase (36). When the ssDNA of the Rep/ssDNA co-crystal structure is added to the super-imposed model of the SF2 HCV helicase, a potential DNA contact with a region between motifs Ia and II of HCV helicase is also observed (37). In this region, the bacterial nucleotide excision repair helicase UvrB contains a β hairpin domain that inserts between the two DNA strands and is responsible for physically separating the strands from one another (38). Furthermore, this motif was identified as an Iron-Sulfur metal binding domain in the XPD helicase family to which BACH1 belongs (39) (Figure 7). The Met-299 position of BACH1 resides immediately adjacent to the second of four conserved cysteine residues that compromise the metal binding domain between the Walker A (motif I) and Walker B (motif II) motifs (Figure 7). It remains to be determined

if BACH1 protein binds iron through its conserved metal binding domain as reported for the *Sulfolobus acidocaldarius* XPD protein (39). The notion that mutations in the Iron–Sulfur (Fe–S) domain of BACH1 are likely to be biologically important is supported by the observation that the mutation A349P in BACH1, immediately adjacent to the last cysteine of the Fe–S cluster, was identified in a patient with severe clinical symptoms of FA (11). Mutation of Met-299 to isoleucine may perturb the local structure of BACH1 protein to dramatically alter the enzyme's ability to hydrolyze ATP.

Although the helicase efficiency (i.e. number of base pairs separated per ATP hydrolyzed) is not known for BACH1, it seems likely that the increased motor ATPase function of the M299I variant improves its ability to unwind the backbone-modified DNA substrates. Moreover, the BACH1-M299I helicase is able to unwind longer DNA duplexes without modifications better than BACH1-WT (R. Gupta, S. Sharma, K. M. Doherty, J. A. Sommers, S. B. Cantor and R. M. Brosh, Jr, unpublished data), suggesting a potential significance for the improved helicase activity of the M299I variant.

The M299I allele was originally identified as a germline BACH1 coding sequence change in a patient with early-onset breast cancer (1). Demonstration that the BACH1-M299I variant helicase has a gain of function for unwinding chemically damaged DNA substrates raises the question of how this mutation might impact the function of BACH1 *in vivo* and contribute to a cancer phenotype. Our findings set a new standard that a mutation in a helicase gene of a patient may enhance the enzyme's ability to unwind damaged DNA. It is conceivable that elevated helicase activity may lead to a clinical syndrome different from that of loss of helicase activity. In the future, it will be important to characterize FA mutations to determine which class they belong. A model for BRCA1 checkpoint function at sites of DNA damage involves the assembly of a BRCA1 super-complex containing BARD1, BACH1, BRCA2 and Rad51 that is critical for the S phase checkpoint by inhibiting DNA synthesis at late-firing sites of replication initiation (40). Dysregulated helicase activity of BACH1-M299I may be detrimental to proper maintenance of the S phase checkpoint.

A second mechanism whereby the elevated helicase activity of BACH1-M299I would be disadvantageous for genomic stability is based on the hypothesis that BACH1 serves to remove Rad51 from DNA (41), as demonstrated for SRS2 helicase (42). Although depletion of either BRCA1 or BACH1 disrupts HR to a similar degree, BRCA1 deficiency negates Rad51 foci formation after DNA damage whereas Rad51 foci form in BACH1-deficient cells. A model was proposed that BACH1 functions downstream of Rad51 to release Rad51 from DNA strands to complete HR (41). BRCA1, on the other hand may down-regulate BACH1 helicase activity to prevent the untimely displacement of Rad51 from the ssDNA filaments until the strand exchange step of HR is complete. BRCA1 deficiency would result in uncontrolled and premature Rad51 dissociation by BACH1 helicase prior to the fulfillment of strand exchange. A testable model is that BACH1-M299I helicase activity fails to be regulated in an appropriate manner by BRCA1 and disrupts HR by either

displacing Rad51 or the invading third strand of a D-loop intermediate at the inappropriate time. Mechanistic studies of the role of BACH1 with its protein partners in chromosomal stability will lead to further insights toward understanding how their deficiencies lead to tumourigenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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