

CELL BIOLOGY. For the article “The BRCA1-associated protein BACH1 is a DNA helicase targeted by clinically relevant inactivating mutations,” by Sharon Cantor, Ronny Drapkin, Fan Zhang, Yafang Lin, Juliana Han, Sushmita Pamidi, and David M. Livingston, which appeared in issue 8, February 24, 2004, of *Proc. Natl. Acad. Sci. USA* (**101**, 2357–2362; first published February 17, 2004; 10.1073/pnas.0308717101), the authors note that the *x* axis of the left graph in Fig. 2*B* is numbered incorrectly. The corrected figure and its legend appear below.

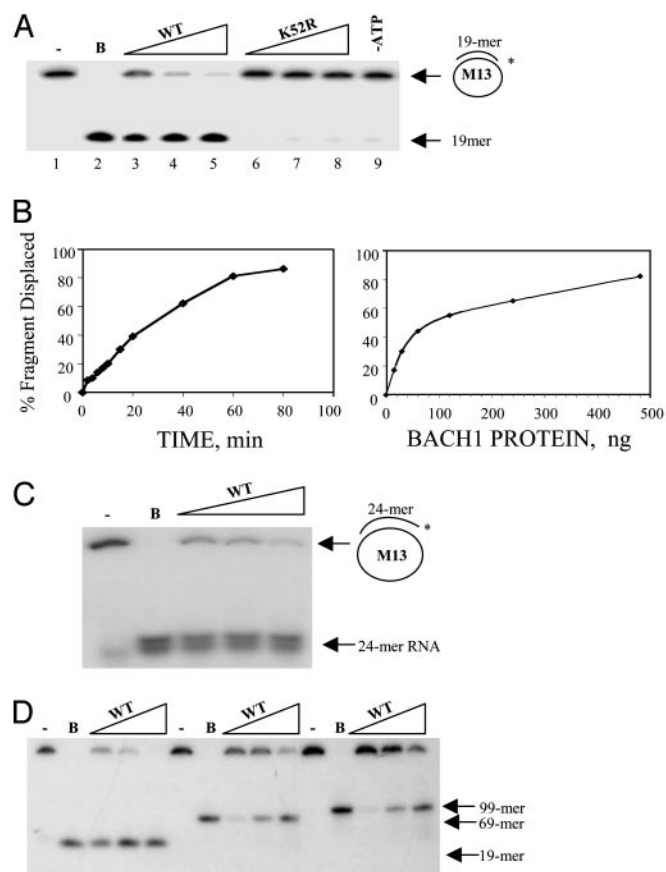


Fig. 2. BACH1 is an ATP-dependent helicase. (A) Increasing amounts of WT and K52R mutant BACH1 were incubated with a DNA helicase substrate containing an annealed radiolabeled 19-nt oligomer (see *Materials and Methods*). Lane 1, annealed substrate (–); lane 2, heat-denatured substrate (B, for boiled); lanes 3–5, BACH1 (60, 180, and 450 ng, respectively); lanes 6–8, K52R BACH1 (200, 400, and 600 ng, respectively); lane 9, WT with no ATP. (B) BACH1 unwinds DNA in a time- and dose-dependent manner. BACH1 protein (150 ng) was incubated with the 19-mer helicase substrate for the indicated times. Independently, increasing amounts of BACH1 (15, 30, 60, 120, 240, and 480 ng) were incubated with substrate for 30 min. (C) Increasing amounts of BACH1 (60, 180, and 450 ng) were incubated with a RNA:DNA helicase substrate and helicase activity was measured. (D) Increasing quantities of BACH1 (60, 180, and 450 ng) were incubated with DNA helicase substrates of increasing partial duplex length, as indicated. In all cases, reaction products were resolved in an 8% native polyacrylamide gel containing 15% glycerol. Results were quantitated by using a Molecular Dynamics STORM PhosphorImager.

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MICROBIOLOGY. For the article “Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection,” by Frederik W. van Ginkel, Jerry R. McGhee, James M. Watt, Antonio Campos-Torres, Lindsay A. Parish, and David E. Briles, which appeared in issue 24, November 25, 2003, of *Proc. Natl. Acad. Sci. USA* (**100**, 14363–14367; first published November 10, 2003; 10.1073/pnas.2235844100), the authors note that the day 4 colonization data of strain EF3030 of the nasal washes have been omitted from Fig. 1*B*. The corrected figure and its legend appear below.

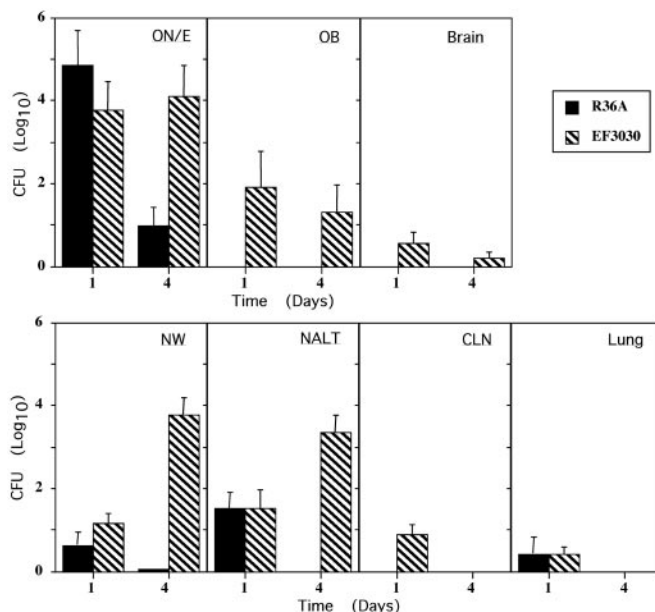


Fig. 1. Nasal delivery of 3×10^6 cfu of either the nonencapsulated R36A strain or the virulent EF3030 strain of *S. pneumoniae* to *xid* mice. The neuronal tissues (ON/E, OB, and brain) and the lymphoid tissues (NALT, CLN, and lungs) were collected, minced, and analyzed for the presence of live pneumococci at 1 and 4 days after nasal challenge. Indicated is the mean of \log_{10} cfu \pm 1 SE. The 0 value on the *y*-axis represents the absence of detectable cfu. Indicated are the mean cfu \pm SE of five mice per group; data are representative of three different experiments.

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MICROBIOLOGY. For the article “Mycobacterial polyketide-associated proteins are acyltransferases: Proof of principle with *Mycobacterium tuberculosis* PapA5,” by Kenolisa C. Onwueme, Julian A. Ferreras, John Buglino, Christopher D. Lima, and Luis E. N. Quadri, which appeared in issue 13, March 30, 2004, of *Proc. Natl. Acad. Sci. USA* (**101**, 4608–4613; first published March 18, 2004; 10.1073/pnas.0306928101), due to a printer’s error, the word “acyltransferase” incorrectly appeared as “acetyltransferase” in the eighth line of the abstract.

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The BRCA1-associated protein BACH1 is a DNA helicase targeted by clinically relevant inactivating mutations

Sharon Cantor^{*†}, Ronny Drapkin^{†‡}, Fan Zhang[†], Yafang Lin[†], Juliana Han[†], Sushmita Pamidi[†], and David M. Livingston^{*§}

^{*}The Dana–Farber Cancer Institute, Harvard Medical School, Boston, MA 02115; and ^{*}Department of Cancer Biology, University of Massachusetts Medical School, Lazare Research Building, 364 Plantation Street, Worcester, MA 01605

Contributed by David M. Livingston, December 31, 2003

BACH1 is a nuclear protein that directly interacts with the highly conserved, C-terminal BRCT repeats of the tumor suppressor, BRCA1. Mutations within the BRCT repeats disrupt the interaction between BRCA1 and BACH1, lead to defects in DNA repair, and result in breast and ovarian cancer. BACH1 is necessary for efficient double-strand break repair in a manner that depends on its association with BRCA1. Moreover, some women with early-onset breast cancer and no abnormalities in either BRCA1 or BRCA2 carry germline BACH1 coding sequence changes, suggesting that abnormal BACH1 function contributes to tumor induction. Here, we show that BACH1 is both a DNA-dependent ATPase and a 5'-to-3' DNA helicase. In two patients with early-onset breast cancer who carry distinct germline BACH1 coding sequence changes, the resulting proteins are defective in helicase activity, indicating that these sequence changes disrupt protein function. These results reinforce the notion that mutant BACH1 participates in breast cancer development.

BRCA1 is a nuclear phosphoprotein with an N-terminal BRING domain and tandem C-terminal BRCT motifs. The latter are prototypical members of a protein fold superfamily present in numerous proteins associated with genome stability control (1). The integrity of these repeats in BRCA1 is critical for its participation in double-strand break repair (DSBR) and homologous recombination (2–5). In this regard, the majority of disease-associated BRCA1 mutations result in a truncated product with loss of the extreme C terminus and one or both BRCT motifs. Clinically relevant missense mutations also exist within each BRCT motif, implying a link between their function and BRCA1-mediated tumor suppression.

We previously identified a helicase-like protein that directly interacts with the BRCA1 BRCT motifs and termed it BACH1, for BRCA1-associated C-terminal helicase (6). The first suggestion that BACH1 might be critical to BRCA1 tumor suppression function was the observation that tumor-predisposing missense and deletion mutations in the BRCA1 BRCT domain, all of which render BRCA1 defective in its DSBR function, also disrupt BACH1 binding to BRCA1 (6). In addition, overexpression of a BACH1 allele carrying a mutation in its ATP binding pocket (Lys-52 → Arg) resulted in a marked decrease in the ability of cells to repair DSBs, suggesting that this mutation operates in a dominant-negative manner. Interestingly, this phenotype depended on a specific interaction between BACH1 and BRCA1 (6). More recently, it was shown that the interaction between BRCA1 and BACH1 depends on the phosphorylation status of BACH1 and that this phosphorylation-dependent interaction is required for DNA damage-induced checkpoint control during the G₂/M phase of the cell cycle (7). Thus, BACH1 likely plays a critical role in DSBR in a manner dependent on its association with BRCA1.

The association of a functional defect in a DNA helicase and either decreased cell viability or disease development is well documented (reviewed in refs. 8–10). Bloom's, Werner's, and Rothmund–Thomson genomic instability disorders all predis-

pose patients to tumor development and are the products of mutant helicase encoding genes (11). In addition, mutations in two helicases, XPB and XPD, have been linked to xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (11); also, certain polymorphisms in XPD are associated with an increased risk of basal cell carcinoma and melanoma (12).

Previously, we detected a potential association between the presence of certain germline BACH1 sequence changes and breast cancer development (6). Two independent germline BACH1 alterations were detected among a cohort of 65 women with early-onset breast cancer. The fact that BACH1 sequence changes exist in a group of early-onset breast cancer patients and not in 200 normal controls led to speculation that BACH1, like BRCA1, can exert a tumor suppression function.

Here, we demonstrate that BACH1 is both a DNA-dependent ATPase and an ATP-dependent DNA helicase that translocates in a 5'-to-3' direction. Importantly, its enzymatic activity was found to be defective in two patients with germline BACH1 coding unit sequence abnormalities who experienced early-onset breast cancer. These findings further support the view that BACH1 has “caretaker”-type tumor suppression activity.

Materials and Methods

Generation of Baculoviruses Expressing BACH1. Full-length WT BACH1 or mutants, P47A, M299I, and K52R (6), were subcloned into the transfer vector, PVL1392 (BD Pharmingen). A BACH1-Bluescript vector was digested with *Apa*I, and the resulting ends were filled in with T4 DNA polymerase. The cDNA was then digested with *Not*I and subcloned into the *Not*I/*Sma*I site of PVL1392. To incorporate a C-terminal FLAG-tag, the BACH1-PVL1392 plasmid was digested with *Bam*H1, and a BACH1 C-terminal fragment was replaced with an identical fragment containing a C-terminal FLAG-tag that was generated by PCR (Table 1, which is published as supporting information on the PNAS web site). Following the manufacturer's protocols (BD Pharmingen), baculoviruses were used to infect High Five cells that were harvested 48 h postinfection. Cell pellets were resuspended in buffer A (10 mM Tris·HCl, pH 7.5/130 mM NaCl/1% Triton X-100/10 mM NaF/10 mM NaPi/10 mM NaPPi). Cells were lysed in the presence of protease inhibitors (Roche Molecular Biochemicals) for 45 min on ice with mild agitation and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was incubated with FLAG antibody resin (Sigma) for 2 h at 4°C. The resin was then washed extensively with 500 mM NETN (50 mM Tris·HCl, pH 7.4/500 mM NaCl/0.5% Nonidet P-40/1 mM EDTA) followed by a 150 mM NETN wash. BACH1 was eluted with 4 μg/ml FLAG peptide (Sigma) in BC100 (25 mM Tris·HCl, pH 7.4/100 mM NaCl/10% glycerol/5

Abbreviation: DSBR, double-strand break repair.

[†]S.C. and R.D. contributed equally to this work.

[§]To whom correspondence should be addressed. E-mail: david.livingston@dfci.harvard.edu.

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mM DTT/0.1% Tween 20) for 1 h. FLAG-BACH1 protein was then dialyzed against BC100 for 2 h, and aliquots were frozen in liquid nitrogen and stored at -80°C . FLAG-tagged BRCA1 encoding virus was the gift of Martin Gellert (National Institutes of Health, Bethesda).

ATPase Assay. The ATPase activity of BACH1 protein was detected by measuring the release of free phosphate during ATP hydrolysis as described (13, 14). All experiments were repeated at least five times.

DNA and RNA Helicase Substrates. Twelve different DNA and RNA oligonucleotides were used to construct the substrates for helicase assays (Table 1). The DNA oligonucleotides were purchased from Invitrogen, and all were complementary to a segment of M13mp18 single-stranded DNA (M13) (New England Biolabs). The RNA oligonucleotides were purchased from Oligos Etc. (Wilsonville, OR). Those RNA oligos longer than 35 nt contained a single 3' deoxynucleotide base.

To study the polarity of unwinding by BACH1, a 92-nt oligomer (M13-92; Table 1) was annealed to M13 DNA, cleaved with *SaI*, and labeled at the resulting 3' ends with [α - ^{32}P]CTP, as described (15) (Table 1). This resulted in linear M13 DNA with a 55-nt fragment annealed to its 5' end and a 38-mer annealed to its 3' end. A second directionality substrate was prepared and tested as described (16).

Oligonucleotides were used to generate partially double-stranded DNA and DNA:RNA duplexes by annealing to M13 DNA. After annealing, the partial duplexes were passed through a MicroSpin S-200 HR column (Amersham Biosciences) to remove free oligonucleotide. The annealed primer was then extended one nucleotide with DNA polymerase I (Klenow fragment) by using 40 μCi of [α - ^{32}P]GTP (1 Ci = 37 GBq). The labeled substrate was purified by three consecutive passages through a MicroSpin G-50 Sephadex column. The 40-nt substrates with 3' and 5' tails were generated as described (16). The RNA-18 and RNA-24 oligonucleotides (Table 1) were labeled with T4 polynucleotide kinase before annealing to either M13 DNA (to generate RNA:DNA hybrid duplexes) or to RNA-68 to generate RNA:RNA duplexes.

Helicase Assays. Helicase activity was measured by detecting the displacement of labeled DNA or RNA oligonucleotide from the partially duplexed substrate. Helicase reactions (20 μl) contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM MgCl_2 , 2 mM DTT, 2 mM ATP, 2% glycerol, 100 $\mu\text{g}/\text{ml}$ BSA, and the indicated nucleic acid substrate. The reaction was initiated with enzyme and incubated at 30°C for 30 min, unless otherwise indicated. The reaction was stopped with 4 μl of stop solution (50 mM EDTA/2% SDS/40% glycerol/0.1% bromophenol blue). Reaction products were resolved by electrophoresis in an 8% native TBE (89 mM Tris base/89 mM boric acid/2 mM EDTA, pH 8.3) polyacrylamide gel containing 15% glycerol.

Antibodies. The polyclonal BACH1 antibody (E67) was generated by immunizing New Zealand White rabbits with a GST-BACH1 fusion protein containing residues 998-1249 of BACH1. Monoclonal antibodies against BACH1 were described previously (6).

Mapping the BRCA1 Binding Domain of BACH1. A full-length *BACH1* cDNA clone was constructed as described (6). To construct C-terminal deletion mutants of the protein, PCR reactions were performed with the BACH1-Forward primer and a series of different reverse primers, HD, C1, C2, C3, and C4-Reverse (Table 2, which is published as supporting information on the PNAS web site). An N-terminal deletion of BACH1 was generated by using the PCR primers N1-Forward and C1-Reverse

(Table 2). PCR products were digested with *NotI/ApaI* and subcloned into the pCDNA3.0 myc-his-tag expression vector (Invitrogen). The chimera protein consisting of residues 961-1008 of BACH1 was generated by PCR using 46-Forward and 46-Reverse primers (Table 2). The PCR product was digested with *BamHI* and *EcoRI* and subcloned into the active loop of the thioredoxin A protein as described (17). A deletion of BACH1 residues 979-1006 was generated by using QuikChange site-directed mutagenesis (Stratagene) with the 28-Forward and 28-Reverse primers (Table 2).

Results

Purification of Recombinant BACH1 from Insect Cells. To determine whether BACH1 is a bona fide helicase, a baculovirus expression system was used to produce BACH1 C-terminal FLAG-tagged recombinant protein. A mutant form of BACH1 (K52R) predicted to be enzymatically inactive (6) was generated in parallel. This conserved residue, when mutated in the XPD and ChlR1 helicases, rendered these proteins inactive (18-20). This same BACH1 mutation disrupted BRCA1-mediated DSBR (6).

The WT and mutant BACH1 proteins were resolved electrophoretically, and the gel was stained to assess protein purity (Fig. 1A). In both the WT and K52R protein lanes, a major band was present at the predicted size of 130 kDa and accounted for >90 -95% of the protein. A faint, faster migrating band of ≈ 45 kDa was also visible in both lanes (see below).

To determine whether the 130-kDa protein is the *BACH1* gene product, we asked by Western blot analysis whether BACH1-specific antibodies recognize this protein. Previously characterized BACH1 monoclonal antibodies (6) specifically reacted with the 130-kDa band (Fig. 1B). Antibodies to another DEAH family member, SMARCAD1 (21), which migrates like BACH1 on SDS polyacrylamide gels, did not recognize recombinant BACH1 (data not shown). Moreover, recombinant FLAG-tagged BACH1 protein bound to a GST-BRCA1 (BRCT) fusion protein and not GST alone in a standard GST pull-down experiment (data not shown). The minor 45-kDa polypeptide also reacted with the BACH1-specific antibody, suggesting that it is a breakdown product of the full-length protein.

ATPase Activity of the Purified BACH1 Protein. Helicases are molecular motors that couple the hydrolysis of ATP to the unwinding of complementary DNA or RNA strands. ATP binding and hydrolysis are prerequisites for the strand separation activity of all known helicases. Therefore, in the absence of any direct evidence that BACH1 is an enzyme, we asked whether the protein could function as an ATPase. The ATPase activity of helicases is generally strongly stimulated by the presence of a nucleic acid cofactor (22). ATPase reaction mixtures containing purified WT or K52R BACH1-FLAG tagged proteins were evaluated by using calf thymus (CT) DNA, circular single-stranded M13 DNA, and supercoiled pCDNA3.0 as cofactors (Fig. 1C). In the absence of DNA, recombinant BACH1 displayed minimal ATP hydrolysis activity. However, its activity was greatly stimulated by CT DNA and M13 single-stranded DNA. Double-stranded plasmid DNA (pCDNA3.0) also stimulated activity, albeit less dramatically. BACH1 ATP hydrolysis was, as expected, a time-dependent process (Fig. 1D), and heat-denatured WT protein was inactive (data not shown). Purified BACH1-K52R lacked ATPase activity and failed to be stimulated by single-stranded DNA (Fig. 1C and D). These results indicate that BACH1 possesses an intrinsic, DNA-dependent ATPase activity.

DNA Helicase Activity of Purified BACH1. To determine whether purified recombinant BACH1 possesses DNA helicase activity, it was incubated with single-stranded circular M13 DNA containing an annealed ^{32}P -labeled 19-mer complementary frag-

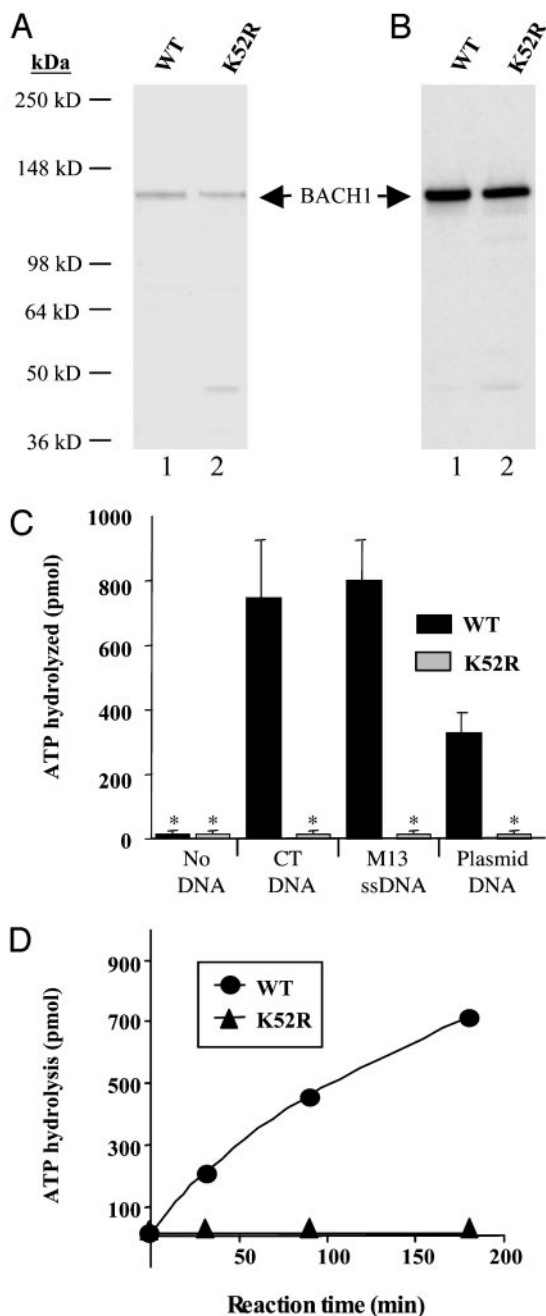


Fig. 1. Purified BACH1 is a DNA-dependent ATPase. (A) FLAG WT and mutant (K52R) BACH1 proteins were purified by FLAG-affinity chromatography from High Five insect cells infected with the corresponding baculoviruses. The proteins were resolved in 4–12% gradient SDS-polyacrylamide gels and stained with Coomassie blue. Lane 1, 500 ng of WT BACH1; lane 2, 500 ng of K52R mutant. (B) Western blot analysis of recombinant proteins. The blot was probed with polyclonal BACH1 antiserum (E67) that recognizes the C terminus of BACH1. Lane 1, 25 ng of WT BACH1; lane 2, 25 ng of K52R mutant. (C) WT BACH1 hydrolyzes ATP in the presence of DNA. Calf thymus (CT), single-stranded (M13), and double-stranded plasmid DNA served as nucleic acid cofactors. WT and K52R mutant BACH1 proteins (600 ng each) were incubated with [γ - 32 P]ATP-containing reaction mixtures supplemented with 2 μ g of the indicated DNA for 60 min at 37°C. ATP hydrolysis was quantitated as described in *Materials and Methods*. The asterisk denotes negligible ATPase activity above background. Results were calculated from experiments performed in triplicate. (D) ATP hydrolysis by BACH1 is approximately linear over time. WT and K52R mutant BACH1 (600 ng each) were incubated in the above-noted [γ - 32 P]ATP- and CT DNA (2 μ g) containing reaction mixture for the times indicated, and the results were determined. Similar results were obtained in at least three independent experiments.

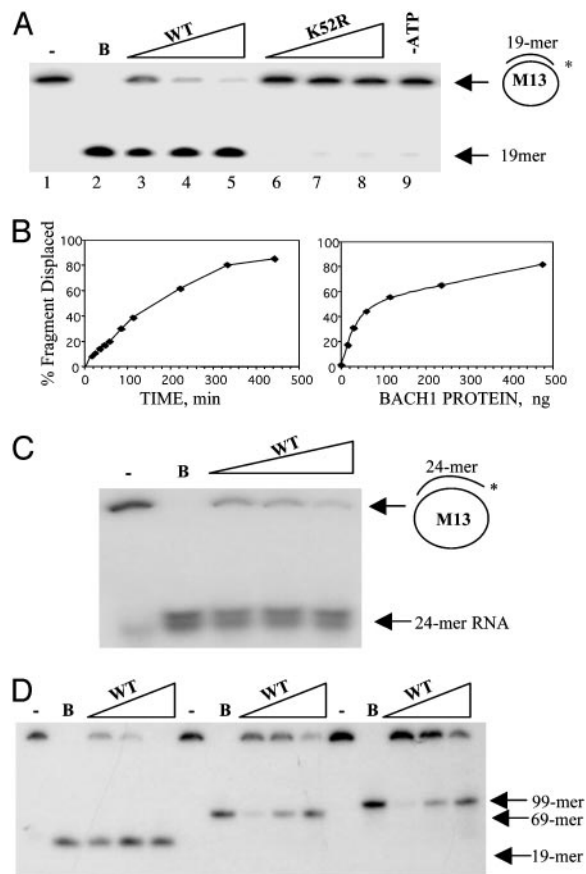


Fig. 2. BACH1 is an ATP-dependent helicase. (A) Increasing amounts of WT and K52R mutant BACH1 were incubated with a DNA helicase substrate containing an annealed radiolabeled 19-nt oligomer (see *Materials and Methods*). Lane 1, annealed substrate (–); lane 2, heat-denatured substrate (B, for boiled); lanes 3–5, BACH1 (60, 180, and 450 ng, respectively); lanes 6–8, K52R BACH1 (200, 400, and 600 ng, respectively); lane 9, WT with no ATP. (B) BACH1 unwinds DNA in a time- and dose-dependent manner. BACH1 protein (150 ng) was incubated with the 19-mer helicase substrate for the indicated times. Independently, increasing amounts of BACH1 (15, 30, 60, 120, 240, and 480 ng) were incubated with substrate for 30 min. (C) Increasing amounts of BACH1 (60, 180, and 450 ng) were incubated with a RNA:DNA helicase substrate and helicase activity was measured. (D) Increasing quantities of BACH1 (60, 180, and 450 ng) were incubated with DNA helicase substrates of increasing partial duplex length, as indicated. In all cases, reaction products were resolved in an 8% native polyacrylamide gel containing 15% glycerol. Results were quantitated by using a Molecular Dynamics STORM PhosphorImager.

ment (Fig. 2A). BACH1 catalyzed the unwinding of the partial duplex in a time- and concentration-dependent fashion (Fig. 2B). As expected, the K52R mutant was inactive in this assay (Fig. 2A). The helicase activity was strictly dependent on the presence of ATP (Fig. 2A, lane 9). Moreover, the addition of EDTA to the reaction inhibited the activity, consistent with a requirement for certain cations in the reaction (data not shown). To determine whether endogenous BACH1 functions as an active enzyme, BACH1 was immunoprecipitated from HeLa cells with a BACH1 polyclonal antibody. Anti-BACH1 immunoprecipitates exhibited both ATPase (data not shown) and helicase activity, whereas control immunoprecipitations did not, suggesting that endogenous BACH1 exists as an active enzyme (Fig. 5, which is published as supporting information on the PNAS web site).

To determine the range of substrates that BACH1 can unwind, its preference for DNA, RNA, and hybrid substrates was examined. An RNA:DNA heteroduplex was constructed by 5' end

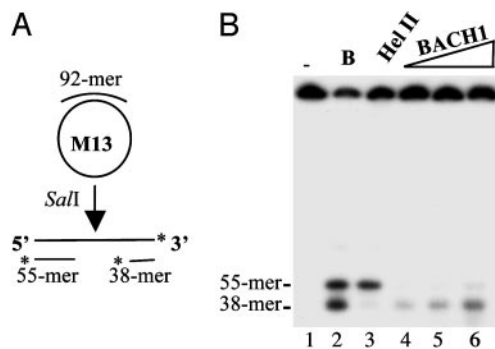


Fig. 3. BACH1 preferentially unwinds DNA in the 5'-to-3' direction. (A) Scheme for helicase directionality assay. A single-stranded 92-mer oligodeoxynucleotide was annealed to M13 DNA and cleaved with *SalI*, and all available 3' ends were radiolabeled by using Klenow polymerase to yield a partially double-stranded substrate comprising 38-mer and 55-mer oligonucleotides annealed to linear M13 DNA. (B) Autoradiogram of an 8% polyacrylamide gel of the products generated by incubating the substrate depicted in A with FLAG-BACH1. Lane 1, substrate alone; lane 2, denatured substrate (B); lane 3, 5 ng of UvrD protein as a 3'-to-5' helicase control; lanes 4–6, substrate plus 60, 150, and 450 ng of FLAG-BACH1.

labeling a 24-nt oligoribonucleotide (RNA-24; Table 1) and annealing it to single-stranded M13 DNA, as described in *Materials and Methods*. Addition of BACH1 resulted in the displacement of the oligomer from the DNA template (Fig. 2C). This data indicates that BACH1 can unwind not only DNA:DNA substrates but also RNA:DNA hybrid substrates. Under conditions where a known RNA helicase [e.g., RNA helicase A (23)] catalyzes efficient strand separation of a double-stranded RNA substrate, BACH1 was inactive (data not shown), consistent with the fact that BACH1 lacks structural motifs that are unique to RNA helicases (24).

To better understand the enzymatic characteristics of BACH1 helicase, we attempted to measure the optimal length of a DNA duplex that could be unwound by BACH1 after annealing to M13 DNA. Oligonucleotides of 18, 68, and 98 nt (Table 1) were annealed to the template and labeled as described. Increasing quantities of BACH1 protein catalyzed the unwinding of all of these partial duplex substrates (Fig. 2D). Although BACH1 was able to completely unwind the short duplex (19-mer), it only partially unwound the longer substrates (69-mer and 99-mer). Some helicases (e.g., Werner's and Bloom's enzymes) require an accessory factor for maximal unwinding of longer duplexes (25–27). Because BACH1 interacts directly with BRCA1, we asked whether full-length BRCA1 or its BRCT repeat-containing region serves that purpose for BACH1. However, addition of WT recombinant BRCA1 or the GST-BRCT fusion protein had no measurable effect on BACH1 helicase activity (data not shown).

The BACH1 Helicase Acts in the 5'-to-3' Direction. Most helicases translocate along one strand of a DNA duplex in a single direction, and the particular directionality of unwinding is an intrinsic feature of each helicase. To determine the directionality of the BACH1 helicase, a 92-nt oligomer (M13–92; Table 1) was annealed to M13 DNA and cleaved as described in *Materials and Methods*. The product was a linear M13 DNA molecule bearing a 55-mer fragment annealed to its 5' end and a 38-mer annealed to its 3' end (Fig. 3). BACH1 preferentially displaced the 38-mer fragment in a concentration-dependent manner, indicating translocation in the 5'-to-3' direction with respect to the strand to which the enzyme is bound. The product of the *Escherichia coli* UvrD gene, Helicase II, served as a 3'-to-5' control in these experiments and selectively displaced the 55-mer (Fig. 3, lane 3).

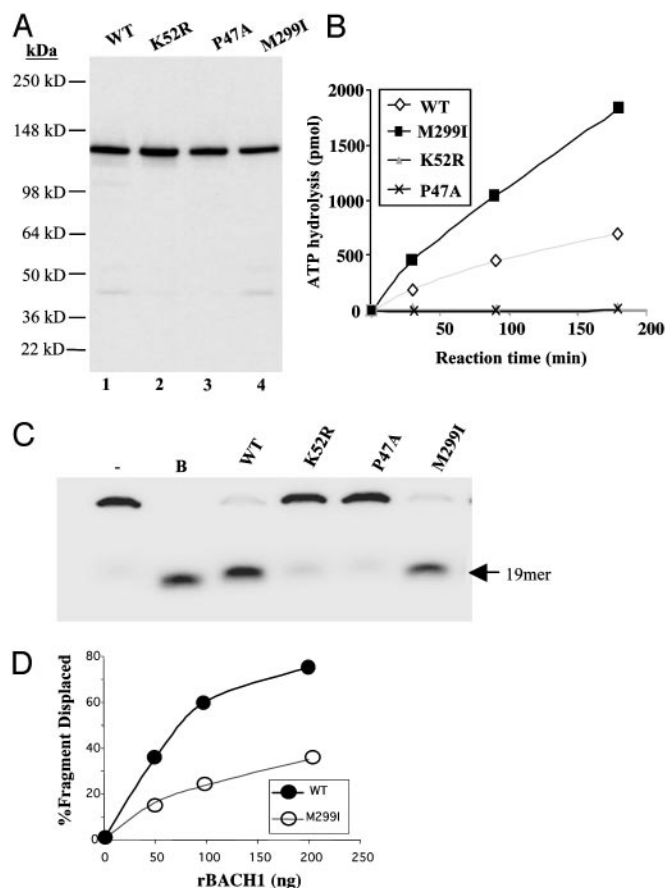


Fig. 4. Germ-line sequence changes in BACH1 disrupt BACH1 helicase activity. (A) Baculovirus-expressed WT, K52R, P47A, and M299I BACH1 species were purified simultaneously and resolved on a 4–12% SDS-polyacrylamide gradient gel. Western blot analysis was performed by using a BACH1 specific polyclonal antibody. (B) Time course of ATPase activity of WT and BACH1 mutant species. (C) Helicase activity of WT and mutant BACH1 proteins (200 ng each) using a 19-mer-containing substrate. (D) Equivalent amounts of WT and M299I BACH1 were determined by quantitative Western blot analysis and protein measurements. Equal quantities of the two BACH1 proteins were incubated with a 99-mer-containing substrate. Results were quantitated with the assistance of a Molecular Dynamics STORM PhosphorImager and represented as percent released ^{32}P -99-mer. Similar results were obtained in three independent experiments.

In an effort to confirm these results, a second distinct directionality substrate was prepared as described in *Materials and Methods*. As with the initial directionality substrate, BACH1 displayed 5'-to-3' polarity (data not shown). Thus, the BACH1 helicase operates as a 5'-to-3' unwinding protein.

Characterization of Clinically Relevant BACH1 Mutations. We previously identified two females with early-onset breast cancer who carried germline sequence changes in the *BACH1* coding region and normal genotypes for *BRCA1* and *BRCA2* (6). The potential impact of these sequence changes on BACH1 helicase activity was assessed. BACH1 WT, K52R, and the P47A and M299I proteins identified previously (6) were synthesized as FLAG-tagged recombinant proteins. Western blot analysis of these BACH1 species revealed that all migrated as intact polypeptides (Fig. 4A). By using equivalent quantities of WT and mutant BACH1 protein, comparative ATPase and helicase assays were performed. The P47A and K52R species exhibited no detectable ATPase or helicase activity (Fig. 4B and C). These findings demonstrate that a clinically relevant sequence change in the

BACH1 coding region results in a catalytically defective protein. Interestingly, under similar conditions, the M299I *BACH1* protein displayed modestly elevated ATPase activity compared to WT (Fig. 4B). Surprisingly, its apparently elevated ATPase activity did not result in increased helicase activity. Although M299I could effectively unwind a 19-nt duplex, it could not effectively unwind longer duplexes compared to WT *BACH1* (Fig. 4D). Thus, both sequences altered *BACH1* proteins function. The P47A change results in complete loss of function, whereas the M299I change perturbs the ability of the protein to unwind longer substrates.

Discussion

BACH1 functions together with *BRCA1* to mediate proper and efficient repair of double-strand breaks. How these proteins cooperate to execute this function is unclear. Here, we show that *BACH1* is intimately involved in DNA metabolism by virtue of its role as a DNA-dependent ATPase and DNA-dependent helicase. Links between abnormal DNA helicase function and human disease are well established (11). The causative mutations in Bloom's, Werner's, and Rothmund-Thomson syndromes have all been mapped to genes encoding DNA helicases (28). All three syndromes are characterized by chromosomal instability, suggesting that DNA helicases are important caretakers of the human genome (29). Furthermore, mutations in the DNA helicases, *XPB* and *XPD*, can result in xeroderma pigmentosum, Cockayne syndrome, or trichothiodystrophy (11, 12) and are the result of defects in transcription-coupled and nucleotide excision repair pathways (11, 30). The association of the *BACH1* helicase with *BRCA1* and their mutually dependent execution of DSB repair is consistent with a similar role for *BACH1* helicase activity in DNA repair and the maintenance of genomic integrity. The extensive homology between *BACH1* and other DNA helicases known to function in DNA repair and genomic stability control (*ChlR1*, *XPB*, *SUVI*, and *DinG*) (11, 20, 31, 32) further supports this notion.

BRCA1 maps at 17q21 and *BACH1* at 17q22. The frequent documentation of allelic losses in the 17q21–q22 region coupled with failure to detect *BRCA1* mutations in the same breast carcinomas suggests that this chromosomal region harbors an additional breast cancer susceptibility gene (33). Loss of heterozygosity (LOH) in 17q is also a frequent event in ovarian cancer (34). Based on its function and chromosomal location, *BACH1* is such a candidate. We previously screened 65 women with early-onset breast cancers for germline *BACH1* aberrations (6) and detected two distinct heterozygous missense alterations (P47A and M299I) affecting the *BACH1* helicase domain. These alterations were absent among 200 healthy controls and, therefore, are unlikely to represent common polymorphisms. The P47A substitution occurred in a family with a strong history of breast and ovarian cancers and is associated with *BACH1* protein instability (6). Of interest, the analogous proline residue in *XPD* is a critical residue for DNA repair activity (35).

The development of a standard *in vitro* helicase assay made it possible to determine whether the P47A and M299I sequence changes are associated with a defect in *BACH1* helicase activity. The P47A substitution occurs within the highly conserved ATP-binding pocket of *BACH1*. The effects of this change on *BACH1* helicase activity were profound, resulting in complete loss of function of both ATPase and helicase activities. It is unlikely that this defect is a consequence of protein misfolding because the mutant protein is fully soluble and can still interact with *BRCA1* (S.C., unpublished data).

The M299I substitution occurs between helicase domain Ia and II of *BACH1* at a nonconserved residue. Incorporation of this substitution in *BACH1* resulted in ATPase activity that was higher than that seen with WT *BACH1*. However, although on short partial DNA duplexes M299I exhibited comparable activity to that of WT *BACH1*, on longer substrates M299I was less efficient. The

observation that a mutation could confer increased ATPase activity on a DNA helicase is an unexpected result, but not one without precedent. Zhang *et al.* (36) reported a mutation in *E. coli* DNA helicase II (*uvrD*) that increased the ATPase and helicase activities of the protein. This mutant exhibited elevated sensitivity to UV and methyl methanesulfonate (MMS), an alkylating agent that causes DNA lesions. One possible explanation for the effects of this DNA helicase II mutation is that increased ATPase activity could uncouple the repair synthesis reaction orchestrated between DNA polymerase I and DNA helicase II.

In the case of *BACH1*, the results are consistent with several possibilities. First, the increased ATPase activity might result in an abnormal helicase that unwinds short duplexes rapidly but cannot coordinate its activity on longer duplexes, analogous to what was observed with the aforementioned DNA helicase II mutation. In particular, the M299I protein might carry out futile ATP hydrolysis that is not coupled to translocation. Second, many helicases operate as multimers composed of identical subunits arranged as dimers or hexamers (37). These observations have led to the suggestion that the active forms of most helicases are often oligomeric (38, 39). Thus, one possibility is that the M299I mutation perturbs the ability of *BACH1* to form active, higher-order complexes. We have observed by gel exclusion chromatography that recombinant WT FLAG-*BACH1* (an ≈ 130 -kDa polypeptide) migrates as an ≈ 500 -kDa, enzymatically active species (R.D. and D.M.L., unpublished data). This finding raises speculation that *BACH1*, too, operates as a multimer in certain settings. In keeping with this notion, native *BACH1* can be isolated in at least two forms; (i) as a megadalton size complex that contains *BRCA1* and *BARD1*, and (ii) as a 500-kDa complex that appears to only contain *BACH1* (R.D. and D.M.L., unpublished data). Whether the M299I mutation prevents proper assembly of active *BACH1* is being investigated.

Thus, two patients with early-onset breast cancer and no *BRCA* gene abnormalities carry germline *BACH1* mutations that render the enzymatic function of *BACH1* abnormal. These observations represent direct biochemical evidence that reinforces the hypothesis that *BACH1* can play a role in the suppression of breast and possibly other forms of cancer (6).

In *XPD*, there are clinically relevant mutations that map outside of the helicase domain (40) and do not disrupt its helicase activity. These mutations are associated with loss of other functions such as transcriptional activity (41). A recent study suggests that perhaps a similar development has occurred with *BACH1*. Analysis of certain Finnish breast and ovarian cancer families identified a patient with a novel germline *BACH1* abnormality (3101C \rightarrow T) that results in a proline to leucine substitution at codon 1034 (P1034L) (42). P1034L resides outside of the *BACH1* helicase domain and does not disrupt ATPase or helicase function (S.C. and D.M.L., unpublished data). However, this mutation maps within the *BRCA1*-binding domain described previously (6). Our more recent mapping results indicate that *BACH1* residues 979–1006 are sufficient for *BRCA1* binding *in vivo* (Fig. 6, which is published as supporting information on the PNAS web site). These findings are consistent with a recent report showing that the *BRCA1*–*BACH1* interaction is mediated by a segment of this sequence that requires phosphorylation of serine 990 (7). Hence, P1034L maps outside of the more narrowly defined *BRCA1* interaction domain of *BACH1*. Although P1034L does not compromise *BACH1* enzymatic function, it remains to be determined whether it perturbs any other *BACH1* function, including its ability to participate in a DNA damage response.

Mutations in a *BACH1* homologue in *Caenorhabditis elegans*, *dog-1* (for deletions of guanine-rich DNA), led to germline as well as somatic deletions in genes containing polyguanine tracts (43). Based on these observations, it was proposed that *dog-1* is required to resolve the special secondary structures that occasionally form in

guanine-rich DNA during lagging-strand DNA synthesis. One wonders whether BACH1 performs a similar function in mammalian cells. Failure of this function could result in intragenic deletions and regions of loss of heterozygosity (LOH), lesions known to be associated with cancer cell development.

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