Published in final edited form as:

DNA Repair (Amst). 2014 October ; 22: 104–111. doi:10.1016/j.dnarep.2014.07.013.

Dual DNA-binding domains shape the interaction of Brh2 with DNA

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Abstract

Brh2, the BRCA2 ortholog in the fungus *Ustilago maydis*, harbors two different DNA-binding domains, one located in the N-terminal region and the other located in the C-terminal region. Here we were interested in comparing the biochemical properties of Brh2 fragments, Brh2^{NT} and Brh2^{CT}, respectively, harboring the two different DNA-binding regions to understand the mechanistic purpose of dual DNA-interaction domains. With oligonucleotide substrates to model different DNA conformations, it was found that the substrate specificity of Brh2^{NT} and Brh2^{CT} was almost indistinguishable although avidity was different depending on salt concentration. DNA annealing activity inherent in Brh2 was found to be attributable to Brh2^{NT}. Likewise, activity responsible for a second-end capture reaction modeling a later step in repair of DNA double-strand breaks was found attributable to Brh2^{NT}. Efficient annealing of DNA strands coated with RPA required full length Brh2 rather than Brh2^{NT} suggesting Brh2^{CT} contributes to the activity when RPA is present. Brh2^{NT} and Brh2^{CT} were both found capable of physically interacting with RPA. The results suggest that while the two DNA-binding regions of Brh2 appear functionally redundant in certain aspects of DNA repair, they differ in fundamental properties, and likely contribute in different ways to repair processes involving or arising from stalled DNA replication forks.

Keywords

BRCA2; Rad51; homologous recombination; DNA repair; DNA binding; DNA annealing; second-end capture

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Disclosure statement

The authors confirm that there is no conflict of interest, financial or otherwise, in this work.

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1. Introduction

Repairing damaged DNA by homologous recombination (HR¹) is a universal mechanism for maintaining stability and restoring integrity of the genetic material [1-3]. The HR pathway can process damaged DNA arising from a variety of causes. This includes lesions induced by radiation, oxidation, reactive chemicals, and aberrations from replication-associated events. In eukaryotes, DNA substrates entering the HR pathway are engaged by Rad51, which assembles on exposed single-stranded (ss) regions to form orderly filamentous structures active for homologous pairing and DNA strand invasion. Access of Rad51 to ssDNA is impeded by RPA, which forms tight protective complexes with ssDNA whenever it is exposed, but this inhibition can be overcome by intervention of mediators.

In vertebrates BRCA2 enables delivery of RAD51 to RPA-coated ssDNA [4]. BRCA2 physically interacts with RAD51 and has inherent DNA-binding activity [5]. These combined features serve to mediate RAD51 nucleoprotein filament formation and ready RAD51 for homologous pairing and DNA strand invasion. The DNA-binding domain of BRCA2 (DBD) consists of a tandem array of three OB fold modules that are similar to those found in the ssDNA-binding protein RPA [6]. OB1, the N-terminal proximal OB module, and adjoining helix-rich domain (HD) interact with the intrinsically unstructured polypeptide DSS1, while the distal two modules OB2 and OB3 pack in tandem with the characteristic OB grooves aligning to form the primary DNA interaction site. ssDNA or DNA with ss tails rather than double-stranded (ds) DNA is the preferred substrate for binding.

Brh2 is the BRCA2 ortholog in the fungus *Ustilago maydis* [7, 8]. It seems to be a simpler version of the much larger BRCA2 mammalian protein, having only a single Rad51-interacting BRC motif compared to eight in the human protein, and a DNA-binding domain located in the carboxy terminal domain (CTD) with two rather than three OB modules as present in the mammalian DNA-binding domain (see Figure 1). The first OB fold and adjoining helix-rich domain are highly conserved with almost all of the Dss1-contacting residues apparent in the BRCA2 DBD crystal structure identical or conservatively substituted in Brh2 [9]. A second DNA binding region separate from the OB fold domain was found during biochemical analysis of Brh2 when it was observed that an N-terminal fragment (Brh2^{NT}) deleted of the entire CTD was capable of binding DNA. By deletion analysis the presumed DNA-binding unit or N-terminal binding domain (NBD) was localized to a 144 residue stretch within the Brh2^{NT} [10]. The sequence is poorly conserved bears no significant similarity to BRCA2, and has no hallmark of any other known DNA binding motif. Nonetheless the isolated Brh2^{NT} polypeptide has potent DNA binding activity and is partially redundant with the C-terminal CTD in biological function [11, 12]. Mutant variants of Brh2 with an intact NBD but deleted of the entire CTD can promote

¹The abbreviations used are: BRC, BRCA2-associated Rad51-binding motif; CT, carboxy-terminal; CTD, C-terminal DNA binding domain; DBD, DSS1/DNA-binding domain; ds, double-stranded; HEPES, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); HR, homologous recombination; IRD, infrared dye; 6xHis-tag, hexahistidine-tag; MBP, maltose binding protein; -mer, oligomer of indicated residues; NBD, amino terminal DNA binding domain; NT, amino terminal; NTA, nitrilotriacetate agarose; OB, oligonucleotide/oligosaccharide binding; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; ss, single-stranded; UV, ultraviolet

DNA repair and restore recombination proficiency when cells expressing these forms are challenged with certain types of DNA damaging agents such as ultraviolet light, or gamma radiation. Conversely, mutant variants deleted of NBD but retaining CTD are similarly proficient. However, when DNA replication is stressed by addition of hydroxyurea to the growth medium, a requirement for both NBD and CTD in contributing to deleterious recombination events becomes evident if the RecQ DNA-helicase-system for dismantling and dissolving recombination intermediates is ablated [11].

Biochemical analysis with defined DNA substrates revealed that the preferred conformational forms for Brh2 binding are D-loops, tailed molecules, and branched structures [13]. But Brh2's interplay with DNA includes a repertoire of actions that extends beyond simple binding. Brh2 has an annealing activity that can promote association of complementary strands even in the presence of RPA [13]. This resembles a second end capture reaction between the displaced strand of a D-loop and a complementary single strand as envisioned in models for double-strand break repair [14, 15]. It was also shown to promote a template switch in model reactions involving Rad51-catalyzed D-loops extended by a DNA polymerase to enable DNA synthesis past the site of a lesion [16]. Association of Dss1 with Brh2 is critical for appropriate functional activity in DNA repair and recombination. However, in defined biochemical reactions Dss1 exerts a negative regulatory effect on Brh2's DNA binding and appears to attenuate the association of DNA with the CTD [12, 17]. One might infer from these findings that the nature and dynamics of association of Brh2 with DNA result from an interplay between the two different DNA binding sites.

The question posed at the outset of this investigation was what biological purpose might be served by endowing Brh2 with two structurally different, spatially separated DNA-binding domains. Different scenarios can be envisioned. For instance, one might imagine that a system of two domains with different binding specificities might enable coordinated docking with a structurally complex DNA substrate such as the junction between single- and double stranded conformations of a resected DNA duplex, or the single- and double stranded regions in a D-loop. Or, one might imagine that a two-domain system might promote allosteric coupling to amplify the overall binding affinity as in the case of RPA [18]. Or alternatively, dual domains might function independently but be required for appropriate attachment and positioning to a random coil DNA substrate. Other possibilities are of course conceivable.

Here our aim was to learn more about the fundamental properties of the CTD and NBD in DNA interactions so as to be able to define and distinguish the roles of these different DNA binding domains in Brh2 function. Our approach was to compare properties of purified Brh2 fragments (Brh2^{NT} and Brh2^{CT}, respectively) containing the NBD or CTD domain in assays measuring interplay with DNA. We chose to use the Brh2^{CT} fragment purified without associated Dss1 to simplify interpretation of the results.

2. Methods and materials

2.1 Protein purification and manipulation

Brh2^{NT} terminating at residue M551 was purified as a fusion protein with an N-terminal maltose binding protein (MBP)-tag and C-terminal hexahistidine (His)-tag after expression in *E. coli*. Purification involved sequential affinity chromatography on Ni²⁺-NTA (nitrilotriacetate) agarose (Qiagen), amylose resin (New England Biolabs), salt gradient elution from monoQ ion exchange resin, and salt gradient elution from HiTrap heparin-agarose using an AKTA FPLC chromatography system (GE Healthcare). Brh2 and Brh2^{CT} proteins tagged with MBP were purified after obtaining the respective heterodimeric forms in complex with His-tagged Dss1 as described previously [10, 17], then stripping off and discarding the His-Dss1. After co-expression of MBP-Brh2 or MBP-Brh2^{CT} with His-Dss1 in *E. coli*, the heterodimeric forms were purified by sequential affinity chromatography on Ni²⁺-NTA agarose, amylose resin and monoQ resin. Protein complexes were then incubated in buffer A (50mM Tris-HCl, pH 7.5, 200mM KCl, 1 mM DTT, 1mM EDTA, 10% glycerol) containing 20 mM MgCl₂ at 37° for 1 hr then loaded onto a column containing amylose resin. The column was washed with 100 volumes of buffer A plus 20 mM MgCl₂ at 4° overnight. MBP-Brh2 or MBP-Brh2^{CT} was then eluted with buffer A containing 10 mM maltose. The fraction of Brh2 or Brh2^{CT} protein still complexed with His-Dss1 was negligible (< 5%) as determined by binding to Ni²⁺-NTA agarose. RPA was purified as described previously [13]. *E. coli* SSB was purchased from Promega.

Co-precipitation was performed by an affinity-tag pulldown procedure with MBP-tagged proteins and RPA. Mixes (60 µl) containing 830 nM RPA and 830 nM MBP-tagged Brh2, MBP-Brh2^{NT}, or MBP-Brh2^{CT} in buffer B (35 mM MOPS, pH 7.5, 40 mM KCl, 1 mM dithiothreitol, 0.00% NP-40) were incubated at 32°. After 30 min a slurry (40 µl) of amylose resin beads was added and the mixture was left on ice for 10 min with intermittent mixing. Beads were collected by centrifugation, washed four times (60 µl each) in buffer and then bound protein eluted with buffer B (60 µl) containing 10 mM maltose. Samples (12 µl) were taken for SDS-gel analysis. After electrophoresis gels were stained with SimplyBlue SafeStain (Life Technologies),

2.2 DNA dynamics

DNA binding was assayed by electrophoretic mobility shift using ³²P-labeled oligonucleotides by the methods described previously [12, 13]. All DNA concentrations are expressed in molecules, rather than nucleotide unless specifically noted. Reactions (15 µl) were performed with 3.3 nM DNA in buffer R (25 mM HEPES, pH 7.5, 55 mM KCl, 2 mM MgCl₂), at 37° for 15 min. Glutaraldehyde was added to 0.2% and after an additional 10 min 0.1 M Tris-HCl, pH 8.0, was added to quench crosslinking. Products were examined after electrophoresis at room temperature in 1% agarose gels cast in 40 mM Tris-acetate, pH 7.6, 1 mM EDTA. Oligonucleotide substrates design was along the same lines as described in previous studies [12, 13]. Oligonucleotides were labeled at the 5' end with ³²P using T4 polynucleotide kinase and [γ-³²P]ATP, and annealed with appropriate unlabeled oligonucleotides indicated in the scheme below to yield the desired conformation. After annealing the desired oligomers were purified by electrophoresis in polyacrylamide gels.

Images of gels were acquired by phosphorimaging using a Typhoon Phosphorimager (Molecular Dynamics). The fraction of DNA in complexes was determined using ImageQuaNT software (Molecular Dynamics).

Oligonucleotide substrates were prepared by annealing the following combinations: (1) ss ^{-32}P -labeled strand A. (2) ds ^{-32}P -labeled A annealed with unlabeled strand B. (3) 3' ss tail- ^{-32}P -labeled strand A annealed with unlabeled strand D. (4) 5' ss tail -unlabeled strand C annealed with ^{32}P -labeled strand B. (5) fork ^{-32}P -labeled strand B annealed with strand E.

A. 5'-TAA ATT GTA AGC GTT AAT ATT TTG TTA AAA TTC GCG TTA AAT TTT TGT TAA ATC AGC TCA TTT TTT AAC CAA TAG GCC GAA ATC GGC AAA ATC CCT TAT A-3'; B. 5'-TAT AAG GGA TTT TGC CGA TTT CGG CCT ATT GGT TAA AAA ATG AGC TGA TTT AAC AAA AAT TTA ACG CGA ATT TTA ACA AAA TAT TAA CGC TTA CAA TTT A-3'; C. 5'-AA ATT GTA AGC GTT AAT ATT TTG TTA AAA TTC GCG TTA AAT TTT TGT TA-3'; D. 5'-TAA CAA AAA TTT AAC GCG AAT TTT AAC AAA ATA TTA ACG CTT ACA ATT T-3'; E. 5'-GAC GCT GCC GAA TTC TGG CTT GCT AGG ACA TCT TTG CCC ACG TTG ACC CGA ATC AGC TCA TTT TTT AAC CAA TAG GCC GAA ATC GGC AAA ATC CCT TAT A-3'

DNA annealing was performed using ^{32}P -labeled ss 100mer strand A and unlabeled ss 100mer strand B as substrates. Reactions (15 μl) in buffer R containing 3.3 nM strands A and B (with or without RPA) and Brh2 protein or fragment, respectively, in three separate microdroplets pipetted onto the sidewall of a 1.5 ml microcentrifuge tube were started by a brief spin to combine the components. After 15 minutes at 32° reactions were quenched by addition of 20 fold excess unlabeled ss A strand together with proteinase K, SDS, and EDTA. Samples were loaded onto polyacrylamide gels (10%) in Tris-glycine buffer, pH 8.8, for electrophoretic separation. Gels were imaged and bands were quantified as above.

Second-end capture reactions (30 μl) were performed in S buffer (35 mM MOPS pH 7.5, 40 mM KCl, 1 mM DTT, 0.001% nonidet NP-40. Brh2 or truncated form (330 nM) was added to reaction tubes containing a suspension (equivalent to 1 μl of commercial stock) of streptavidin magnesphere paramagnetic particles (Promega Biotech) that had been soaked sequentially in 5% nonfat milk, followed by 150 nM 3'-biotinylated ss 60mer 1st strand (Eurofins MWG/Operon) pre-annealed with a tracer strand (3.3 nM 5' IRD800 ss 50mer) to serve as a 1st strand reporter. 1st strand sequence was based on nucleotides 103-162 from bacteriophage ϕX174 , and tracer strand was based on the complementary ϕX174 sequence over residues 132-81. After assembly of protein-bound complexes (32°, 15 min), particles were collected and washed three times (50 μl) with buffer, resuspended in S buffer (30 μl) then mixed with heterologous 2nd strand, 3.3 nM 5' IRD700 ss 60mer based on nucleotides 42-101 from plasmid pBluescript II SK+ (Stratagene). After 15 min at 32° particles were collected and washed once. Bound complexes were stripped off by heating at 100° in SDS-sample buffer, and composition was analyzed after electrophoresis in 10% polyacrylamide gels containing SDS. Brh2 and truncations were detected by staining (Simply Blue Safe stain, Life Technologies). IRD700 and IRD800 labeled DNAs were visualized using the far-infrared fluorescence LI-COR Odyssey platform (LI-COR Biosciences).

3. Results

3.1 Influence of DNA conformation on binding activity

In previous work it was noted that full-length Brh2 supported homologous recombination and survival after UV irradiation, and in addition promoted killing if cells lacking the Bloom's syndrome DNA helicase ortholog Blm [19] were challenged with the DNA replication stressor hydroxyurea [11]. On the other hand Brh2 variants deleted of either the NBD or CTD were still competent for homologous recombination and survival after UV irradiation, but unlike native Brh2 did not contribute to cell death by hydroxyurea poisoning in the absence of Blm [11]. These findings were interpreted to mean that Brh2 with only one DNA-binding domain could act to enable HR repair of lesions induced by UV light, presumably single-stranded gaps, but that Brh2 with the natural configuration of both binding domains became pathological in the milieu of complex unresolved structural intermediates resulting from DNA replication stress.

To assess the binding capabilities of the two different DNA-binding domains we tested full length Brh2 and polypeptides Brh2^{NT} and Brh2^{CT}, each deleted of either the CTD or NBD, in gel mobility shift assays using oligonucleotides as substrates to model different types of DNA conformations. In the past we have analyzed DNA binding interactions of Brh2 purified in association with its regulator Dss1, which together form a tight heterodimeric complex [12, 17]. However, because Dss1 attenuates the DNA binding activity of Brh2, we wished to examine binding properties without its interference. So for comparative purposes in this study we used only Dss1-free proteins (Figure 1). Our aim was to identify any structural feature in DNA preferred by one DNA-binding domain over the other that might give a clue to how the different domains function together. For substrates, we utilized oligonucleotides to model the following conformation-- single-stranded, double-stranded, forked (splayed arms), and tailed duplexes with a protruding 5' or 3' single-stranded stretch. Forked DNA was the preferred substrate in DNA binding for all three protein preparations (Figure 2). Following this in order of preference were ss and 3'-tailed DNA, then 5'-tailed DNA, and finally dsDNA. Titrations revealed that in the protein levels for half-maximal binding as well as for overall yields in formation of complexes, Brh2^{CT} resembled full length Brh2 slightly more than Brh2^{NT}. Notwithstanding this observation, the general impression is that Brh2^{NT} and Brh2^{CT} are remarkably similar in both capacities for DNA binding as well as in substrate preference.

3.2 Influence of salt on DNA binding activity

The stability of protein-nucleic acids complexes often decreases with increasing ionic strength because electrostatic interactions between protein and DNA play an important role in binding[20]. We tested the effect of increasing salt on formation of DNA-protein complexes to ask if there might be any differences between the Brh2 variants. We found that Brh2^{NT} was able to form complexes with ssDNA at higher concentrations of KCl or MgCl₂ than Brh2^{CT} (Figure 3). This could indicate that electrostatic interactions play a more dominant role in the binding of Brh2^{CT} to DNA than in the binding of Brh2^{NT}. The CTD of Brh2^{CT} contains the highly conserved helix-rich domain and OB folds 1 and 2 [21], which are similar in structure to the OB folds of RPA. As RPA's binding to ssDNA is highly

sensitive to salt [22], the current observations are in line with those findings. The structure of the NBD is not yet known although it is highly basic ($pI = 11.5$) raising the possibility that binding with DNA is promoted through electrostatic interactions. Given that protein folding prediction programs (e.g., [23]) indicate the NBD to be partially folded but certainly less dense in folded structure than the Brh2^{CT} (Figure 1), it might be supposed that electrostatic interactions would predominate the Brh2^{NT} protein interplay with DNA. Thus, it is not clear what feature of the NBD might promote its lower sensitivity to salt. It is notable that complex formation with the full length Brh2 was more sensitive to salt than Brh2^{NT}. This was unexpected given that both NBD and CTD contribute to Brh2's DNA binding activity. One might have supposed the binding activity of full Brh2 to be the same as Brh2^{NT}, the more salt resistant binding domain, if the domains interacted with DNA independently. Such suppressive interaction as observed could indicate negative cooperativity imposed on the NBD by the carboxy-terminal region of the protein.

3.3 DNA annealing activity in Brh2

In earlier studies we found that Brh2 promotes annealing of complementary DNA strands as well as complementary strands coated with RPA [13]. To determine the source of annealing activity we tested Brh2^{NT} and Brh2^{CT} individually. The assay for annealing employed labeled complementary oligonucleotide 100mers as substrates and a polyacrylamide gel-based electrophoretic system capable of separating ss100mers from annealed ds100mers so that the extent of product formation could be gauged. With this system Brh2^{NT} was as effective as full length Brh2 in promoting annealing, while Brh2^{CT} appeared devoid of annealing activity (Figure 4A). Annealing reached a maximum at a Brh2 or Brh2^{NT} protein concentration of 16 nM. As the individual complementary strands were present at 3.3 nM each, or 660 nM total nucleotide, this would correspond to one protein monomer per ~40 nucleotides, or approximately two protein molecules accommodated per ss100mer. The extent of annealing remained at this plateau level when the protein concentration was increased by twenty-fold. This is in contrast to bell-shaped-curve mode of annealing noted with Rad52 protein in which the rate and extent of annealing become impaired with increasing protein after a certain stoichiometric ratio of protein to DNA is attained [24].

When the DNA substrates were pre-bound with RPA, the full-length protein was capable of promoting annealing although a higher level was required to achieve the maximal yield. Brh2^{NT} had slight activity and was able to promote a marginal level of annealing, but Brh2^{CT} was devoid of activity and even slightly depressed the spontaneous annealing reaction (Figure 4B). When DNA was prebound with the prokaryotic single strand binding protein SSB, the full length Brh2 was unable to promote annealing, suggesting the requirement for specific Brh2 contacts with the cognate RPA. When both Brh2^{NT} and Brh2^{CT} were added together in the same reaction annealing was no better than the slight activity of Brh2^{NT} alone (Figure 4C). The findings imply that an element in the carboxy terminal region of Brh2 protein is important for interacting with RPA-bound ssDNA, and that it likely cooperates with Brh2^{NT} in the context of the full-length protein to enable annealing of the complementary single strands.

3.4 Second-end capture

In DSB repair the process initiates when a protruding single strand generated by resection from one broken DNA arm commences DNA strand invasion with a recipient homologous sequence to form a D-loop recombination intermediate. The invading single strand is then extended with some DNA synthesis driving migration of the D-loop. The other DNA arm, the second end, is then captured and brought into register to pair with the displaced strand of the D-loop, or with the invading strand, if it dissociates from the D-loop. This completes rejoining the ends created by the DSB.

It was demonstrated in a previous study that Brh2 could promote second-end capture in a model reaction that measured annealing of the displaced strand of a D-loop and a complementary sequence [14]. However, since complementary strands anneal spontaneously and since the rate of annealing can be accelerated by means independent of protein contact, we wished to evaluate the ability of Brh2 to promote second-end capture and the contribution to the activity by NBD and CTD in a manner that did not rely on complementary strand annealing. Hence, we used a bead-based pull-down procedure to test whether Brh2 or Brh2^{NT} and Brh2^{CT} variants bound to ssDNA immobilized on beads could capture a second, non-complementary ssDNA molecule. This assay has the virtue of being an authentic measure of protein-mediated DNA-DNA interaction.

Full length Brh2 was active in this assay, indicating that the protein was capable of associating simultaneously with two heterologous DNA molecules. In view of this finding and given the two DNA binding sites present in Brh2, it seemed possible that NBD and CTD might cooperate to promote second-end capture. If so, then it might be predicted that neither Brh2 fragment would exhibit activity by itself. However, the results showed that Brh2^{NT} was capable of promoting the second-end capture reaction, while Brh2^{CT} was inactive (Figure 5). Although we cannot exclude the possibility that both NBD and CTD collaborate in promoting second-end capture in the context of the full-length protein, these observations suggest that the NBD alone can accommodate two different DNA strands.

3.5 Brh2-RPA interaction

The conclusion from the above annealing analysis that the carboxy terminal region of Brh2 likely works together with the amino terminal region within the context of the full-length protein to promote annealing of RPA-coated complementary single strands suggests that both regions interact with RPA. We tested this notion of physical interaction with a co-precipitation assay. Brh2, Brh2^{NT}, or Brh2^{CT}, each labeled with an MBP-tag was incubated with purified RPA in reaction mixtures containing buffer and salt but no DNA. After addition of amylose resin to bind all the Brh2 or Brh2 terminal fragment, the composition of the bound and unbound fractions was determined (Figure 6). As evident by the co-precipitated RPA70 and RPA32 subunits, Brh2, Brh2^{NT}, and Brh2^{CT} were all capable of forming stable complexes with RPA. These results show that Brh2^{NT} and Brh2^{CT} independently interact with RPA.

4. Discussion

Three primary conclusions can be drawn from this study of DNA-Brh2 interactions. First, NBD and CTD, the two different DNA-binding regions within Brh2 display a remarkable similarity in preference for DNA substrates, but differ in avidity with increasing salt. Second, the functional activity of the intermolecular reactions annealing and second end capture is inherent within the Brh2 NBD. Third, annealing of DNA coated with RPA requires contributions from both Brh2^{NT} and Brh2^{CT}.

As demonstrated in this current study, the DNA-binding preferences of both Brh2^{NT} and Brh2^{CT} are remarkably similar. This seems surprising given that the two regions share no obvious structural similarity. Furthermore, there seems to be no synergistic enhancement of binding activity exhibited by full-length Brh2 compared to Brh2^{NT} or Brh2^{CT} alone. Thus, it appears there is no strong support for the notion that a difference in specificity might enable coordinated binding to a complex substrate. Nor is there support for the notion that there might be reinforced binding through conformational coupling. Rather, the DNA-binding potential of the full-length protein seems to be a reflection of the activities of each domain as if contributed independently. The near equivalence in specificity might help explain the apparent functional redundancy in DNA repair that is revealed when one or the other DNA binding domain is removed, but the purpose of such redundancy is not clear. The one notable difference in binding conditions was the elevated salt stability observed with Brh2^{NT} compared with Brh2^{CT}. Possibly this reflects some stepwise choreography in domain association with DNA, or release from it.

As similar as the two domains might be in binding preference, they are not functionally equivalent in promoting the annealing and second end capture reactions. These activities are delegated to the NBD only. Both reactions are pairing processes and while the details of how they work are not clear, they certainly could be operating through the same basis. The second-end capture reaction assay was designed to detect interaction between two different DNA strands only if a common protein component was capable of mediating the pairing. Presumably this means that the NBD binding surface can accommodate two different heterologous DNA strands. If this were the case, the local increase in concentration occurring upon association of two strands would strongly favor annealing if the strands were complementary. An alternative interpretation that protein-protein interactions mediate annealing might also be considered. It was determined in the analysis that annealing reached a maximal extent at a Brh2 concentration corresponding to a stoichiometry of about two protein monomers per 100mer oligonucleotide. This might suggest that each DNA strand of a pair of complementary strands must be occupied by at least one Brh2 monomer for a favorable annealing event and raises the possibility that protein-protein interactions might mediate annealing. Indeed, earlier work supported the notion that Brh2 might form dimers [25]. However, the protein-protein interaction region was mapped to the Dss1-interacting region in Brh2^{CT}. So there is no evidence at present to suggest protein-protein interaction through the Brh2^{NT} region, which is responsible for the annealing activity. An alternative notion is that each complementary strand is bound or wrapped by Brh2 positioning bases to extend outwards thus favoring nucleation of base pairing. More sophisticated methodology

such as has been developed with Rad52 [24] and RecO [26] proteins will be required to establish the mechanism of Brh2-promoted annealing.

Unlike the case with naked DNA, annealing of RPA-coated DNA requires full length Brh2 implying that Brh2^{CT} contributes to the reaction. However, it is not yet clear in what capacity Brh2^{CT} contributes. Both Brh2^{NT} and Brh2^{CT} physically interact with RPA, so presumably each forms a unique protein-protein interface with RPA. Perhaps Brh2^{CT} remodels RPA in some way to enable annealing by the NBD within Brh2^{NT}, but it cannot be ruled out that DNA binding by the CTD plays a role in the reaction.

Studies with the *U. maydis* system demonstrated that Dss1 binding to Brh2 has a profound negative effect on DNA-binding activity of the CTD [17]. Removal of Dss1 activates the DNA-binding potential of Brh2, while addition of Dss1 inhibits it [12]. The current work with Brh2 leaves no doubt that the CTD free of Dss1 has strong DNA binding activity. So it would seem that Dss1 provides a servomechanism for modulating Brh2's DNA binding capacity through its association. However, the paradoxical finding remains that while Dss1 physically interacts with the CTD to regulate DNA binding activity, the CTD appears dispensable for biological function. As shown previously certain mutant forms of Brh2 deleted of the entire CTD retain near normal capacities for DNA repair and recombination [27, 28]. Apparently the NBD can substitute for most recombination-function needs in the event the CTD is removed.

A similar situation prevails in the mammalian system where it was found using synthetic BRCA2 constructs prepared by fusing different domains that abolishing the DBD had little impact on HR so long as the ability to associate with PALB2 was maintained [29]. PALB2 is a partner of BRCA2 that physically interacts with the N-terminal region of BRCA2 and that has its own intrinsic DNA binding domain [30]. The DNA binding characteristics of PALB2 are similar to those of the Brh2 NBD in that the preferred substrates are branched structures with protruding single stranded arms and D-loops [31, 32]. There is also an endogenous annealing activity in PALB2 [31]. There is no evidence as yet to suggest that there is a second cryptic DNA-binding domain in the N-terminal region of BRCA2 as in the case with Brh2 and the NBD. But a similar kind of dualistic behavior as noted with Brh2 seems apparent from the studies with the synthetic BRCA2 fusions [29]. HR is functional in the absence of the DBD so long as there is PALB2 interaction, and conversely, HR is functional when PALB2 is removed so long as the DBD remains. No structure of the PALB2 DNA binding domain is available. By truncation analysis and biochemical assay the DNA binding region of PALB2 has been localized to certain tracts of residues [31], but no obvious binding fold is apparent from bioinformatics analysis, nor is there sequence similarity to the OB folds of the BRCA2 DBD. The PALB2 class of proteins appears confined to metazoans; it is absent in *U. maydis* and other fungi. Thus, it seems not unreasonable to suppose that PALB2 could well provide a DNA-binding activity analogous to the Brh2 NBD, but unlike the NBD DNA-binding function of Brh2, it has evolved to be physically separated from BRCA2.

Finally, we note that the one currently recognized situation with Brh2 in which the presence of both DNA-binding domains appears necessary for function is revealed under pathological

circumstances. In the absence of Blm helicase, cell death brought about by HU-induced replication stress is dependent upon HR. Deletion of the gene for Brh2 suppresses the inviability, at the same time causing loss of DNA repair and recombination proficiency. Indeed, inactivation of any of the known genes required for HR function suppresses the inviability[11]. However, suppression of the inviability is also observed in *blm brh2* mutant cells still active in HR through expression of a Brh2 mutant variant lacking one or the other DNA-binding domain. Thus the relationship between HU-induced inviability in *blm* mutant cells proficient in recombination is puzzling. The generality of the suppression with loss of any HR function suggests the phenomenon is not due to some peculiar feature of Brh2. It could be that our assays for recombination proficiency are simply too unsophisticated in biological readout to reflect the full spectrum of Brh2's action in HR. Perhaps recombination intermediates resulting from attempted repair of stalled replication forks differ structurally from those formed after radiation damage and demand the full capability of the recombination machinery. More sophisticated assays or detection methods employing DNA substrates modeling stalled replication forks might be successful in helping gain insights into the contributions of the dual DNA binding domains to HR function.

Acknowledgments

We thank laboratory members Drs. Jeanette Sutherland and Milorad Kojic for discussion and helpful suggestions. This work was supported by grants GM042842 and GM079859 from the NIH.

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Fig. 1.

Brh2 protein and truncated variants. A. SDS-gel analysis of Brh2, Brh2^{NT}, and Brh2^{CT} proteins used in this study is shown in the top panel. B. Shown schematically to scale with the histogram below is Brh2 featuring the Rad51-interacting BRC element, the N-terminal DNA binding domain NBD, and the C-terminal domain CTD containing the helix rich domain HD, the tandem OB folds, OB1 and OB2 with the emergent tower domain. Probability of folding Brh2 with respect to amino acid residue as determined by FoldIndex [23] is depicted in the histogram.

Fig. 2.

DNA-binding specificity of Brh2 and variants. A. DNA-binding reactions were performed with Brh2 or truncated variants using ^{32}P -labeled DNA substrates as described in the Methods. Images of typical gels are shown. Protein concentrations (μM) in lanes as follows: a, 0; b, 2; c, 4; d, 10; e, 20; f, 100; g, 200. B. Bands were quantified and background signal (lane a, no protein added) for each set was subtracted from the signal for complexes in each lane to obtain a value for the fraction DNA bound. Results are presented graphically in the panel on the right.

Fig. 3.

Effect of salts on DNA binding. DNA-binding reactions with varying concentrations of salt were performed with 100 μ M Brh2 or truncated variants using 32 P-labeled ssDNA as substrate. Bands were quantified and values presented graphically below the corresponding gels. A. Effect of KCl, concentrations (mM) as follows: a, 20; b, 40; c, 80; d, 120; e, 160; f, 200; g, 260. B. Effect of MgCl₂, concentrations (mM) as follows: a, 0; b, 0.5; c, 1; d, 2; e, 6; f, 8; g, 10. Error bars indicate standard deviations from three determinations.

Fig. 4. DNA-annealing promoted by Brh2 and variants. A. Annealing reactions with Brh2 or variants were performed with ^{32}P -labeled 100mer oligonucleotide as described in Methods. Protein concentrations (μM) were added as follows: a, 0; b, 8; c, 16; d, 32; e, 65; f, 128; g, 256. B. RPA (92 μM) or SSB (45 μM) was incubated with both complementary strands for 15 min on ice before mixing together with the indicated proteins.

Fig. 5.

Second-end capture. A. The assay is illustrated schematically in the top panel. B. Reactions were initiated by adding Brh2 or variant (330 μ M) to magnetic beads pre-coated with ssDNA and reporter oligomer. After incubation at 37° for 10 min bead were collected and washed, then mixed with 2nd strand oligomer. After a second incubation beads were collected, washed, and stripped of components by holding in SDS-sample buffer at 100° for 2 min. Components were separated by electrophoresis in polyacrylamide gels and analyzed after protein staining and fluorescence imaging.

Fig. 6.

Physical interaction between Brh2 and RPA. Reactions containing RPA and MBP-tagged Brh2, Brh2 truncation, or no protein control were performed as described in Materials and Methods. After addition of amylose resin to capture the MBP-tag, the composition of the bound and unbound fractions was determined by SDS-gel electrophoresis. lane 1, Brh2; lane 2, Brh2^{NT}; lane 3, Brh2^{CT}; lane 4, no protein.