Potential Role for the BLM Helicase in Recombinalional Repair via a Conserved Interaction with RAD51*

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Bloom's syndrome (BS) is an autosomal recessive disorder that predisposes individuals to a wide range of cancers. The gene mutated in BS, BLM, encodes a member of the RecQ family of DNA helicases. The precise role played by these enzymes in the cell remains to be determined. However, genome-wide hyper-recombination is a feature of many RecQ helicase-deficient cells. In eukaryotes, a central step in homologous recombination is catalyzed by the RAD51 protein. In response to agents that induce DNA double-strand breaks, RAD51 accumulates in nuclear foci that are thought to correspond to sites of recombinational repair. Here, we report that purified BLM and human RAD51 interact in vitro and in vivo, and that residues in the N- and C-terminal domains of BLM can independently mediate this interaction. Consistent with these observations, BLM localizes to a subset of RAD51 nuclear foci in normal human cells. Moreover, the number of BLM foci and the extent to which BLM and RAD51 foci co-localize increase in response to ionizing radiation. Nevertheless, the formation of RAD51 foci does not require functional BLM. Indeed, in untreated BS cells, an abnormally high proportion of the cells contain RAD51 nuclear foci. Exogenous expression of BLM markedly reduces the fraction of cells containing RAD51 foci. The interaction between BLM and RAD51 appears to have been evolutionarily conserved since the C-terminal domain of Sgs1, the Saccharomyces cerevisiae homologue of BLM, interacts with yeast Rad51. Furthermore, genetic analysis reveals that the SGS1 and RAD51 genes are epistatic indicating that they operate in a common pathway. Potential roles for BLM in the RAD51 recombinational repair pathway are discussed.

Germline mutations in the BLM gene give rise to Bloom's syndrome (BS), a rare disorder associated with stunted growth, facial sun sensitivity, immunodeficiency, fertility defects, and a greatly elevated incidence in the occurrence of a wide range of cancers (1). BLM encodes a 159-kDa protein that is a member of the RecQ family of DNA helicases (2). This highly conserved family of proteins is required for the maintenance of genomic stability in all organisms (3). In humans, five RecQ helicases have been identified. In addition to BLM, mutations in two other genes encoding RecQ helicases in humans have been associated with disease conditions: WRN and RECQ4, being defective in Werner's syndrome and Rothmund-Thomson syndrome, respectively (4, 5). Werner's syndrome is primarily associated with premature aging, and Rothmund-Thomson syndrome with skin and skeletal abnormalities, but both disorders also give rise to an elevated incidence of cancers (6, 7). All RecQ family members contain a catalytic helicase domain that comprises seven highly conserved motifs found in many DNA and RNA helicases (3). Outside of this helicase domain, the RecQ family proteins show little sequence conservation. In BLM, these non-conserved domains are located both N- and C-terminal to the helicase domain and comprise ~650 and 450 amino acids, respectively. It is likely that these non-conserved domains are important in functionally differentiating the roles of the different RecQ helicases within the cell by either providing additional enzymatic functions, such as the exonuclease activity dependent upon the N-terminal domain of WRN (8, 9), or by mediating interactions with other proteins (10).

Cells from BS patients display genomic instability, the diagnostic feature being an increase in the frequency of sister chromatid exchanges (SCEs) (11). Sonoda et al. (12) recently demonstrated that SCE formation requires homologous recombination (HR). Moreover, chicken BLM heterologous cells display elevated SCE levels that are partially dependent on RAD54 (13). BLM therefore seems to function in the regulation of HR events during replication. Consistent with this notion, mutations in SGS1 or rgh1, the budding and fission yeast RecQ homologues, respectively, also give rise to excessive recombination events (14–16). In rgh1 mutants, inhibition of DNA replication, in particular, stimulates this excessive recombination (16). Further evidence of a role for RecQ helicases in HR comes from the finding that RecQ, Sgs1, BLM, and WRN can all disrupt four-way junctions, a structural mimic for the Holliday junction intermediate formed during HR (17–20). Moreover, both BLM and WRN can promote branch migration of Holliday junctions (19, 20).

The general mechanism for repair of DNA double-strand breaks (DSBs) by HR has been conserved in evolution. The central step involves the pairing of the DSB with homologous sequences to facilitate the exchange of DNA strands. In bacteria, this process is mediated by RecA, which forms a nucleoprotein filament on single-stranded DNA formed as a result of exonucleolytic processing of the DSB to generate single-stranded DNA tails. This nucleoprotein filament facilitates the search for homologous sequences and provides a structure within which DNA strand exchange occurs. In eukaryotes, essentially the same reaction is performed by RAD51, which is structurally related to RecA (21–24). In Saccharomyces cerevi-
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S. cerevisiae, the RAD51 gene is a member of the RAD52 epistasis group, and rad51 mutants display defects in mitotic and meiotic recombination and sensitivity to ionizing radiation, highlighting the key role that RAD51 plays in both HR and recombinational repair of DNA strand breaks (25). Studies on RAD51 in higher eukaryotes have been hampered by the fact that mice with a targeted disruption of the RAD51 gene die early during embryogenesis (26, 27). However, studies with early mouse embryos suggest that loss of RAD51 renders cells sensitive to ionizing radiation (26). Additionally, RAD51 mutant chicken cells, maintained by expression of human RAD51 (hRAD51) under the control of a regulable promoter, accumulate chromosomal breaks following repression of hRAD51 synthesis (28). This suggests a role for hRAD51 in the repair of DNA breaks in undamaged cycling cells, most likely those arising during DNA replication. Treatment of mammalian cells with agents that induce DSBs, such as ionizing radiation, induces localization of Rad51 to nuclear foci that are believed to correspond to multi-protein complexes engaged in recombinational repair (29). However, the precise composition of these recombinational repair centers is unknown.

In this study, we have examined the possibility that the Bloom’s syndrome gene product functions in the RAD51 recombinational repair pathway given the defects in recombination displayed by BS cells. BLM and hRAD51 were found to directly interact in vitro and co-immunoprecipitate from nuclear extracts. Consistent with these data, BLM forms nuclear foci, a subset of which, co-localize with hRAD51. Furthermore, the degree to which these two proteins co-localize to nuclear foci increases in response to ionizing radiation. The interaction between BLM and hRAD51 appears to have evolutionarily conserved since Sgs1 physically associates with yeast RAD51, and genetic analysis reveals that the genes encoding these two proteins are epistatic.

**Experimental Procedures**

Cell Lines—The SV40-transformed normal human fibroblast cell line, WI-38 (obtained from ATCC), was used as a representative normal human cell line. The GM08505 cell line is an SV40-transformed fibroblast cell line from a BS patient (obtained from NIGMS, National Institutes of Health, Bethesda, MD) and contains a BLM homozygous frameshift mutation at residue 739 resulting in premature truncation of the protein (2). PSNF5 cells were derived from a clone of GM08505 cells stably transfected with pcDNA3/BLM. Western blotting using an anti-BLM antiserum (IHC33) (10) confirmed that BLM was stably expressed by this line. Functional complementation of the BLM phenotype was assessed by SCE frequency analysis which showed that PSNF5 cells have a near-normal SCE frequency. Derivation and characterization of these cells will be described elsewhere.2 All cell lines were routinely cultured in a minimal essential medium supplemented with 10% fetal bovine serum. HeLa S3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

**Bacterial and Yeast Strains**—The Escherichia coli strain EGY48 (F thiA leu2 traD46 trec trpC1 thiI46 hsdR17 rpsL22 galK1 endA1 lacY1 supE44 recA1 gyrA96 thiI11811649 recF183 endA1 relA1 leu2-3, 112 his500158 L31) was used in all experiments. The yeast strain used was BY4741 (MATa his3-11, 15, leu2-3, 112 lys2-801 ura3-52 can1-100). All yeast prototrophic (leu2-3, 112 his3-11, 15, ura3-52) transformants were purified using appropriate selective plates.

**Northern Analysis**—Total RNA was isolated from yeast cells by a modification of the method of Chomczynski and Sacchi (30). RNA (20 μg) was treated with RNase-free DNase I (Boehringer Mannheim) and electrophoresed on 1.5% agarose gels containing 2.2 M formaldehyde. Gels were air-dried and transferred to positively charged nylon membranes (GeneScreen Plus nylon, Pharmacia Biotech) for hybridization. Membranes were UV cross-linked (Stratalinker; Stratagene) and prehybridized at 42 °C in high-salt hybridization buffer (0.5 M NaCl, 50 mM Na2HPO4, 5 mM EDTA, 1% SDS) for 1 h. Membranes were hybridized with nick-translated probes labeled with [32P]dCTP with a random priming kit (Boehringer Mannheim). Filters were washed at 55 °C in high-salt wash buffer (0.15 M NaCl, 50 mM Na2HPO4, 5 mM EDTA, 1% SDS) for 15 min. Blots were exposed to Kodak XAR film at −70 °C.

**Immunofluorescence Analysis**—Intracellular localization of BLM and hRAD51 was visualized using the methodologies described previously (10) using BFL103 and anti-RAD51 antibodies, respectively, from exponentially growing WI-38 cells or cells that had been arrested in S phase using aphidicolin. Immunolabeling was performed on cells grown on coverslips using mouse monoclonal antibodies against BrdUrd conjugated to Cy3 (Sigma) and anti-mouse IgG conjugated with rhodamine (Sigma). DNA was visualized by Hoechst staining.

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RESULTS

BLM and hRAD51 Directly Interact via the N- and C-terminal Domains of BLM—The helicase domain in BLM, in common with the human WRN and RECQ4 proteins, is flanked by relatively large N- and C-terminal domains (3) that are likely important in functionally distinguishing these larger members of the RecQ helicase family. To analyze the role of these domains in BLM, we performed a yeast two-hybrid screen of a HeLa cDNA library using the C-terminal domain (residues 966–1417) of BLM as bait. One positive clone, isolated independently three times, was found to encode hRAD51. The interaction appeared specific since hRAD51 did not interact with two nonspecific control bait proteins (Fig. 1A). Further mapping revealed that the C-terminal 150 amino acids of BLM (residues 1267–1417) were sufficient to mediate an interaction with hRAD51. Since only a fragment of BLM had been used in the two-hybrid screen, and interactions using this system could in principle be mediated by adaptor proteins, we used Far Western analysis with purified recombinant BLM and hRAD51 to determine if the full-length proteins were able to interact directly with each other. Far Western analysis revealed that the full-length proteins could interact (Figs. 1B and 2B) and that the interaction was specific since hRAD51 did not interact with either of two control proteins used in this assay, GST and MBP (Fig. 1C and data not shown). Using affinity-purified GST fusion peptides containing various portions of the C-terminal domain of BLM, the final 100 residues, representing amino acids 1317–1417 of BLM, were identified as being sufficient to mediate an interaction with hRAD51 (Fig. 1C), thus providing independent confirmation of the location of the hRAD51 interaction domain on BLM identified by the yeast two-hybrid system (Fig. 1A).

To investigate the possibility of additional hRAD51 interaction domains on BLM, Far Western analysis was performed between hRAD51 and a GST fusion protein containing the first
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Table I

Quantitation of the proportion of cells containing BLM and hRAD51 foci in untreated, irradiated, or aphidicolin-treated WI-38 cells (A) and quantitation of nuclear foci containing both BLM and hRAD51 in untreated, irradiated, or aphidicolin-treated WI-38 cells (B).

| A. Untreated | γ-Irradiation | Aphidicolin |
|--------------|---------------|-------------|
| Cells containing BLM foci | 45% | 78% | 83% |
| Cells containing hRAD51 foci | 22% | 93% | 93% |
| Mean no. of BLM foci/cell | 4.4 (0–29) | 17.2 (0–48) | 9.3 (0–22) |
| Mean no. of hRAD51 foci/cell | 10.1 (0–38) | 27.2 (0–70) | 11.7 (0–80) |

| B. Untreated | γ-Irradiation | Aphidicolin |
|--------------|---------------|-------------|
| No. of BLM and hRAD51 co-localizing foci/cell | | |
| 0 | 84% | 76% | 59% |
| 1–5 | 15% | 15% | 38% |
| >5 | 1% | 9% | 3% |
| Mean no. of BLM and hRAD51 co-localizing foci/cell | 2.1% | 4.7% | 2.5% |

212 amino acids of BLM. hRAD51 was also able to specifically interact with this protein (Fig. 2A), indicating that BLM contains at least two domains with which hRAD51 can independently associate with (Figs. 1C and 2A). In contrast to full-length BLM, a purified recombinant mutant protein, BLM-NC, which consists of residues 213–1266 of BLM and therefore lacks both the N- and C-terminal hRAD51 interaction domains on BLM, was unable to bind hRAD51. This suggests that no additional portions of BLM interact with hRAD51 (Fig. 2B).

BLM and hRAD51 Form a Complex in Vivo and Co-localize to Nuclear Foci in Response to DNA Damage—In response to DNA DSBs generated by ionizing radiation, hRAD51 localizes to nuclear foci that are thought to correspond to sites of ongoing repair (Table I, part A) (29). BLM also forms nuclear foci in undamaged normal cells (Table I, part A) that have been shown previously to correspond to PML nuclear bodies (33–35). In an asynchronous population of WI-38 cells, a human fibroblast cell line derived from a normal individual, the proportion of cells containing BLM foci and the average number of BLM foci/cell was found to increase in response to ionizing radiation (Table I, part A) (Fig. 3). We therefore examined the possibility that BLM might co-localize with hRAD51 foci in response to ionizing radiation. In a population of untreated WI-38 cells, 16% of the cells contained a mean of 2.1 co-localizing BLM and hRAD51 foci/nucleus (Table I, part A) (Fig. 3). Three hours after 10 Gy of γ-irradiation, these figures increased significantly such that 24% of the population contained a mean of 4.7 co-localizing BLM and hRAD51 foci/nucleus (Fig. 3 and Table I). The demonstration that BLM and hRAD51 directly interact in vitro and co-localize to nuclear foci suggested that these two proteins might exist as part of a multiprotein complex in vivo. To investigate this further, we analyzed whether BLM and hRAD51 could be co-immunoprecipitated from WI-38 nuclear extracts. In extracts prepared from unirradiated or γ-irradiated WI-38 cells, we were unable to reproducibly co-immunoprecipitate BLM using anti-hRAD51 antibodies (data not shown). One possible explanation for this is that only a maximum of 24% of these cells were observed to contain BLM and hRAD51 co-localizing foci (Table I, part B). We therefore sought a treatment that would enrich for the number of cells containing BLM and hRAD51 co-localizing foci. BLM is cell-cycle regulated and is most highly expressed in the S and G2/M phases of the cell cycle and is absent from G1 cells (36). Synchronization of cells in early S phase using aphidicolin resulted in an increase in the number of cells containing BLM and hRAD51 foci (Table I, part A) and, concomitantly, a nearly 3-fold increase (41% versus 16%) in the proportion of cells containing BLM and hRAD51 co-localizing foci (Table I, part B). Using nuclear extracts prepared from these aphidicolin-treated cells, we were able to readily immunoprecipitate BLM using anti-

Fig. 3. BLM and hRAD51 co-localize to nuclear foci in response to γ-irradiation. Indirect immunofluorescence of BLM (red) and hRAD51 (green) in untreated and irradiated WI-38 cells as indicated on the left. In the merged image, co-localization is seen as yellow. The panel labeled DNA indicates the position of the nucleus, as judged by Hoechst staining.

Fig. 4. BLM and hRAD51 exist as a complex in nuclear extracts. Western blot for the detection of BLM in immunoprecipitates of nuclear extracts prepared either from untreated or aphidicolin-arrested WI-38 cells. The immunoprecipitating antibody used was either anti-hRAD51 or pre-immune sera as indicated above. The position of BLM is indicated on the right.
hRAD51 antibodies suggesting that BLM and hRAD51 exist as a complex in vivo at least under certain circumstances (Fig. 4).

A High Proportion of Undamaged BS Cells Contain Sites of Recombinational Repair—The precise mechanism by which hRAD51 forms nuclear foci is unknown. We therefore determined if BLM was required for hRAD51 to localize to nuclear foci. In the absence of exogenous damage, 84% of GM08505 cells, a fibroblast cell line from a BS patient, contained hRAD51 nuclear foci (Fig. 5A). In contrast, in an asynchronous population of PSNF5 cells, a clone derived from GM08505 cells that expresses the BLM cDNA, only 30% of the cells contained hRAD51 nuclear foci (Fig. 5A). A similar proportion of hRAD51 nuclear foci-containing cells was also observed in exponentially growing WI-38 cells (Table 1, part A). In undamaged normal cells, RAD51 foci have been shown to form predominantly in S phase (37). Therefore, one possible explanation for the difference in the proportion of GM08505 and PSNF5 cells containing hRAD51 foci might be an altered cell cycle distribution. However, fluorescence-activated cell sorting analysis of propidium iodide-stained GM08505 and PSNF5 cells revealed that the two cell lines had comparable proportions of cells in G1, S, and G2 phases of the cell cycle (Fig. 5B). Furthermore, the high proportion of GM08505 cells containing hRAD51 foci suggested that hRAD51 foci can form outside S phase. To confirm this, we pulse-labeled GM08505 and PSNF5 cells with BrdUrd. Indirect immunofluorescence with anti-BrdUrd and anti-hRAD51 antibodies revealed that hRAD51 foci were clearly evident in non-S phase cells in both GM08505 and PSNF5 cells (Fig. 5C). Together, these data indicate that hRAD51 foci can form in the absence of BLM and suggest that, in BS cells, DNA lesions (possibly DSBs) are formed that activate the hRAD51-dependent stress response resulting in a constitutively high level of hRAD51 foci.

The S. cerevisiae RecQ Homologue, Sgs1, Also Interacts with Rad51—Since both RecQ helicases and Rad51 have been conserved through evolution, and given that RecQ helicase mutants in yeast and humans give rise to defects in genetic recombination, we addressed the question of whether Sgs1, the S. cerevisiae homologue of BLM, interacts with yeast Rad51 in the two-hybrid system. Sgs1, like BLM, also contains N- and C-terminal domains that flank the core helicase domain (3). Although the C-terminal domains of Sgs1 and BLM share little sequence homology, the C-terminal domain (residues 978–1447) of Sgs1 was found to specifically interact with yeast Rad51 (Fig. 6A). A region within this domain containing residues 1299–1447 was sufficient to mediate an interaction with yeast Rad51 (Fig. 6A), whereas a fragment containing residues 1319–1447 of Sgs1 was not able to interact with Rad51. This indicates that amino acid residues 1299–1318 of Sgs1 are important for the interaction with Rad51.

We next investigated the possibility that the SGS1 and RAD51 genes are epistatic. Mutants that lack either Sgs1 or Rad51 are viable but are sensitive to agents that perturb DNA replication or certain DNA damaging drugs. In particular, sgs1 mutants are sensitive to the ribonucleotide reductase inhibitor, hydroxyurea (HU), and the methylating agent, methyl methanesulfonate (MMS) (Fig. 6, B and C), as has been found by other workers (38, 39). rad51 mutants also showed a similar magnitude of sensitivity to HU and MMS (Fig. 6, B and C). However, deletion of both the SGS1 and RAD51 genes did not appear to have any additive or synergistic effect on either HU or MMS sensitivity, suggesting that these two genes are epistatic. Together, these data suggest that Sgs1 and Rad51 function as a complex in a common pathway.

**DISCUSSION**

The underlying cellular defects that give rise to BS remain unclear. Cytogenetic analyses of BS cells suggest that either homologous recombination occurs at an increased frequency or that recombination events are aberrantly processed. In this study, we have shown that BLM, the product of the gene defective in BS, forms a complex with the recombination protein hRAD51. This provides the first direct molecular link between BLM and the pathway for HR in human cells.

We have presented several lines of evidence that BLM and hRAD51 interact directly via residues in the N- and C-terminal domains of BLM, consistent with the finding that BLM and hRAD51 form a complex in nuclear extracts. Despite the lack of sequence homology between BLM and Sgs1, outside of the helicase domain, the C-terminal domain of Sgs1 was found to interact with yeast Rad51, indicating that the organization of functional domains within these two RecQ helicases has been conserved to some extent. The evolutionary conservation of an interaction between RecQ helicases and Rad51 implies that these two classes of proteins together perform a fundamentally important role during DNA metabolism.

The finding that SGS1 and RAD51 are epistatic is in agreement with the findings of Gangloff et al. (40), who demonstrated that deletion of RAD51 can rescue the synthetic lethality of an sgs1Δsrs2Δ mutant. One interpretation of these data is that RecQ helicases act downstream of RAD51, which is consistent with the known enzymatic properties of these two classes of proteins. RAD51 catalyzes the early steps of HR, namely the pairing of homologous sequences and exchange of DNA strands to form a Holliday junction recombination intermediate (22, 24). Several RecQ helicases, including BLM and Sgs1, can bind to and disrupt such recombination intermediates (17–20). More specifically, we have shown previously that BLM can promote branch migration of Holliday junctions (19). Such an activity could either promote the formation of exten-
sive stretches of heteroduplex DNA or conversely “destroy” recombination intermediates depending on the direction in which the Holliday junction is translocated. Dual, but opposing, effects on RecA-mediated joint molecule formation have been reported to be a feature of the *E. coli* RecQ protein (17). The interaction between hRAD51 and BLM may therefore serve to recruit BLM to sites of recombinational repair. If the role of BLM is to disrupt recombination intermediates by catalyzing reverse branch migration, then loss of BLM would give rise to excessive recombination. Conversely, if the normal role of BLM is to promote branch migration, then the absence of BLM may lead to incomplete or inappropriate processing of Holliday junctions. Such events could give rise to the cytogenetic abnormalities seen in BS cells. It will therefore be of interest to determine how directionality, if any, is imposed on the branch migration activity catalyzed by BLM in vivo. One possibility is that hRAD51 may load BLM onto Holliday junctions in a particular orientation that would then dictate the direction of junction translocation.

The stress response pathway leading to nuclear hRAD51 focus formation is apparently constitutively activated in BS cells, but can be suppressed by ectopic expression of BLM. These data suggest both that BLM is not essential to induce the stress response pathway, and that a basal level of unrepaired lesions persist in the absence of BLM. This is consistent with the fact that BS cells are not hypersensitive to ionizing radiation (41, 42) and indicate that the pathways for repairing the majority of DSBs are essentially intact in BS cells. Similarly, haploid *sgs1Δ* cells do not exhibit hypersensitivity to ionizing radiation (40). However, in diploid yeast, where DNA DSBs may potentially be repaired using either the sister chromatid or the homologous chromosome, deletion of *SGS1* confers x-ray sensitivity (40), implicating Sgs1 in the HR pathway for repair of certain DNA DSBs.

Not all hRAD51 foci contained detectable levels of BLM, suggesting that BLM only acts upon a subset of DNA lesions processed by hRAD51. One possibility is that BLM may process DSBs that occur at replication forks since BLM has been shown to co-localize to replication foci (43). Such a role would be consistent with the expression profile of BLM through the cell cycle and the replication defects seen in BS cells. DNA strand breaks could arise during replication when, for example, the replication fork encounters a single-stranded DNA nick in the template DNA (Fig. 7). The formation of a DSB would result in collapse of the replication fork (Fig. 7). Indeed, it has been postulated that such events occur every cell cycle in the absence of exogenous DNA damage (44–46). The exact mechanism by which replication is re-initiated following formation of a DSB remains to be elucidated. However, it is becoming ap-
parent that replication restart is inextricably linked to recombination (44–46). In bacteria, RecA-mediated strand invasion of the broken arm of the replication fork into the intact chromosome or facilitate resolution of the junction through branch migration (45). In eukaryotes, RAD51 may serve a similar purpose in allowing replication to reinitiate following replication fork collapse (Fig. 7). The resulting Holliday junction would then need to be removed. We suggest that hRAD51 recruits BLM to perform this function, which could be achieved by either branch migrating the junction toward the end of the chromosome or facilitate resolution of the junction through recruitment of Holliday junction resolvases (Fig. 7).

In summary, BS cells show DNA recombination defects, and here we have established an evolutionarily conserved molecular association between RecQ helicases and Rad51 that links BLM with the pathway of HR. Further studies using purified BLM and hRAD51, in addition to other components of the recombinational repair pathway, will, we hope, shed light on the role that BLM plays during this conserved process, and how aberrant recombination can lead to destabilization of the genome and ultimately tumorigenesis.

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