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BLM is an early responder to DNA double-strand breaks

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Abstract

Bloom syndrome (BS) is an autosomal recessive disorder characterized by a marked predisposition to cancer and elevated genomic instability. The defective protein in BS, BLM, is a member of the RecQ helicase family and is believed to function in various DNA transactions, including in replication, repair, and recombination. Here, we show that both endogenous and overexpressed human BLM accumulates at sites of laser light-induced DNA double-strand breaks within 10 s and colocalizes with γH2AX and ATM. Like its RecQ helicase family member, WRN, the defective protein in Werner syndrome, dissection of the BLM protein revealed that its HRDC domain is sufficient for its recruitment to the damaged sites. In addition, we confirmed that the C-terminal region spanning amino acids 1250–1292 within the HRDC domain is necessary for BLM recruitment. To identify additional proteins required for the recruitment of BLM, we examined the recruitment of BLM in various mutants generated from chicken DT40 cells and found that the early accumulation of BLM was not dependent on the presence of ATM, RAD17, DNA-PKcs, NBS1, XRCC3, RAD52, RAD54, or WRN. Thus, HRDC domain in DNA helicases is a common early responder to DNA double-strand breaks, enabling BLM and WRN to be involved in DNA repair.

Keywords: DNA double-strand break repair; BLM protein; RecQ helicase; Laser micro-irradiation; HRDC domain; DT40 cells

Bloom syndrome (BS) is a rare autosomal recessive disorder characterized by sunlight sensitivity, short stature, and a very high incidence of various types of neoplasia [1,2]. Cells from BS patients exhibit chromosomal instability typified by elevated rates of sister chromatid exchanges, insertions, deletions, telomere associations, and quadriradials. The gene mutated in BS, *BLM*, encodes a member of the RecQ helicase family [3]. To date, five human RecQ helicases have been identified, three of which are associated with genetic disorders, namely, BS, Werner's syndrome, and Rothmund–Thomson syndrome; the latter two syndromes are both characterized by premature aging and a predisposition to cancer. Inactivation of any of three helicases resulted in specific cellular defects, which suggests that these RecQ helicases have specialized functions in the cell [4]. The mechanism that results in the hyper-recombination phenotype of BS has not yet been clarified at the cellular level. However,*in vitro* studies suggest that BLM is a DNA structure-specific helicase that functions to resolve specific DNA structures that resemble recombination intermediates and that may arise during the processing of DNA double-strand breaks (DSBs) or daughter-strand gaps [5].

DSBs are a major threat to the cell and the broken ends should be joined properly to avoid subsequent genomic

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instability. The repair of DSBs is usually executed by two major pathways, namely, non-homologous end joining (NHEJ) and homologous recombination (HR). Several sub-pathways of DSB repair may also exist, since many proteins have been reported to be involved in DSB repair and the coordinated functions of these proteins are necessary for successful DSB repair [6,7]. Studies on BLM and WRN, which is encoded by the gene that causes Werner's syndrome, revealed that these proteins are somehow involved in DSB repair; in accordance with this, the loss of function of these RecQ helicases augments the cell's sensitivity to the DNA-damaging agents that directly or indirectly generate DSBs [8–10]. Moreover, both WRN and BLM proteins have been shown to associate with proteins that are involved in HR and NHEJ and it is believed they may function together at sites of DNA damage [9,11–13]. However, although it is known that human BLM (hBLM) interacts with several repair/recombination proteins, including RPA, Rad51, Rad51D, and MLH1 [14–17], as well as with DNA-damage signaling proteins such as ATM [18], the precise role of BLM remains largely unknown.

The recent development of the laser light micro-irradiation system has led to the identification of proteins that accumulate at DNA sites that were damaged by the laser irradiation. For example, TRF2 was shown to be recruited to the damaged sites early after laser micro-irradiation [19]. In addition, WRN was found to associate immediately with the DSB sites after laser irradiation; this association was shown to be mediated by its HRDC (Helicase-and-RNaseD-C-terminal) domain, which is conserved in some of RecQ family helicases including *Escherichia coli* recQ, budding yeast Sgs1, hWRN, and hBLM [20]. The latter study also carefully defined the damage that is produced by different laser light irradiation conditions. Since BLM is part of the human telomeric complex [21] and interacts with WRN [22], we were interested in determining the cellular dynamics of BLM after DNA damage is induced by the laser irradiation system. Here, we report that, in response to laser-induced DNA damage, both endogenous BLM and exogenously expressed EGFP (enhanced green fluorescence protein)-linked BLM are immediately recruited to the sites of irradiation and persist there for more than 6 h. We further show that the C-terminal region spanning amino acid residues 1250–1292 within the HRDC domain of hBLM is essential for its recruitment. The significance of the rapid recruitment of BLM to sites of DNA damage will be discussed.

Experimental procedures

Cell lines and culture. HeLa cells, U2OS cells, and the *BLM*-mutated fibroblasts derived from a BS patient (GM 03498) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2. The various DT40 cell lines used have been described previously [23–30] and were cultured in suspension with RPMI1640 supplemented with 10% FBS and 1% chicken serum.

Plasmid construction. The plasmids harboring *EGFP* conjugated to full-length *BLM*, truncated *BLM*, or *XRCC1* have been described earlier [31,32]. The DNA fragment encoding the HRDC and NLS domains of BLM was amplified by PCR from full-length *BLM* and inserted into the *Xho*I/*Kpn*I sites of the pEGFP-C1 vector to produce the HRDC construct. To make the HRDCΔC construct, we first introduced a *Hin*dIII site at amino acid residue 1293 by using the Quick Change Site Directed Mutagenesis Kit (STRATAGENE). Since a *Hin*dIII site also exists at amino acid residue 1248, when the mutagenized plasmid was digested with *Hin*dIII and self-ligated, it encoded GFP-BLM that bears a deletion spanning amino acids 1250–1292. To make the HRDC construct in which the lysine at amino acid 1270 has been substituted by glutamine (HRDC-K1270Q), the plasmid carrying the full-length *BLM* gene was mutated by using the Quick Change Site Directed Mutagenesis Kit. All mutations were confirmed by DNA sequencing.

Transfection. To transfect cells with DNA, 1–2 μg of DNA was used. Cells were grown in poly-L-lysine-coated glass dishes overnight and transfected with lipofectamine (Life Technology) according to the manufacturer's instructions. After transfection, the cells were incubated for 24–48 h before being subjected to laser light irradiation.

Laser light irradiation and microscopy. Fluorescence images were obtained and processed by using a FV-500 confocal scanning laser microscopy system (Olympus). The microscope is coupled with micro-irradiation facilities to emit 365 or 405 nm laser light as described in detail previously [20,32]. Both 365 and 405 nm laser lights are focused through a 40× objective lens. We mainly used the 405 nm scan laser system for irradiating the cells in the epifluorescence path of the microscope system. The power of the scan laser can be controlled by altering the scanning times and/or the laser power. The scan laser light of 405 nm wavelength delivers within 5 ms around 1600 nW of energy at full power (total energy delivered on the path is 40 μJ for 500 scans), while one pulse of 365 nm laser through F25 filter used in this experiment delivers 2.5 μJ. We used the 405 nm laser at full power only and regulated the dose by changing the scanning times. Thus, cells were incubated on a 37 °C hot plate in glass-bottomed dishes placed in chambers to prevent evaporation and then irradiated. After irradiation, images were captured at 10–20 s intervals for up to 5–10 min. The energy of the fluorescent light at the irradiated site was measured with a laser power/energy monitor (ORION, Ophir Optronics, Israel). The mean intensity of the track in the irradiated cell was obtained after subtracting the background intensity.

Immunolabeling. Cells were incubated for different time periods after irradiation and then fixed with freshly prepared 4% paraformaldehyde for 15 min on ice. After washing, the cells were permeabilized with 0.25% Triton X-100 for 10 min on ice, then treated with blocking buffer for 30 min at room temperature, followed by incubation with a primary antibody for 16 h at 4 °C. Rabbit polyclonal anti-phosphorylated H2AX (1:400, Upstate Biotechnology), goat polyclonal anti-BLM (1:100, Santa Cruz Biotech), rabbit polyclonal anti-ATM (1:200, Rockland, USA), and rabbit polyclonal anti-RecQL1 (1:200) [33] were used. Bound antibodies were visualized by incubating the cells with secondary antibodies, namely, Alexa 488-labeled donkey anti-goat and Alexa 594-labeled chicken anti-rabbit antibodies. The cells were observed with a laser scan confocal microscope.

TUNEL assay. After laser irradiation cells were washed and fixed with 4% paraformadehyde. DNA double-stranded break was detected by terminal deoxynucleotidyl transferase mediated dUTP-biotin end labeling (Trevigen) followed by incubation with avidin-FITC (1:400).

Detection of incorporated BrdU. After laser irradiation, cells were incubated for 60 min and then for 10 min in the presence of 100 μM BrdU. The cells were fixed and treated with 0.2 M HCl for 1 h to denature DNA. Incorporated BrdU was detected with mouse anti-BrdU monoclonal antibody which was visualized with Alexa 594 labeled donkey anti-mouse antibody.

Photosensitization assay using BrdU. For incorporation of BrdU as photosensitizer during laser irradiation, BrdU at a final concentration of 10 μM was added to the medium 16 h before laser irradiation. Cells were irradiated without washing.