BLAP75, an essential component of Bloom's syndrome protein complexes that maintain genome integrity

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Bloom's syndrome (BS) is a rare human genetic disorder characterized by dwarfism, immunodeficiency, genomic instability and cancer predisposition. We have previously purified three complexes containing BLM, the helicase mutated in this disease. Here we demonstrate that BLAP75, a novel protein containing a putative OB-fold nucleic acid binding domain, is an integral component of BLM complexes, and is essential for their stability *in vivo*. Consistent with a role in BLM-mediated processes, BLAP75 colocalizes with BLM in subnuclear foci in response to DNA damage, and its depletion impairs the recruitment of BLM to these foci. Depletion of BLAP75 by siRNA also results in deficient phosphorylation of BLM during mitosis, as well as defective cell proliferation. Moreover, cells depleted of BLAP75 display an increased level of sister-chromatid exchange, similar to cells depleted of BLM by siRNA. Thus, BLAP75 is an essential component of the BLM-associated cellular machinery that maintains genome integrity.

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Introduction

Bloom's syndrome (BS) is a rare human autosomal recessive disorder characterized by growth retardation, immunodeficiency, reduced fertility, sensitivity to sunlight and high risk of developing various types of cancer (German, 1993; Bachrati and Hickson, 2003). Cells derived from BS patients show genomic instability, including a hallmark feature of elevated frequency of sister-chromatid exchange (SCE) (German, 1969; Chaganti *et al*, 1974; German *et al*, 1977;

Poppe *et al*, 2001). The gene inactivated in BS, BLM, is a member of the RecQ family of DNA helicases (Ellis *et al*, 1995), and its product possesses unwinding activity for several types of DNA substrates (Karow *et al*, 1997; Sun *et al*, 1998; Karow *et al*, 2000; van Brabant *et al*, 2000). Mutation in two other human RecQ helicases, WRN and RECQL4, results in Werner's syndrome and Rothmund-Thomson syndrome, respectively, both of which feature genomic instability, predisposition to cancer and early onset of aging (Yu *et al*, 1996; Kitao *et al*, 1999). The findings that RecQ mutations cause three human genomic instability diseases, together with studies of these helicases in other species, indicate that this family of proteins plays essential roles in maintaining genome integrity (Bachrati and Hickson, 2003).

BLM has been found to interact with Topoisomerase III α (Topo III α), an enzyme that relieves the torsional stress created during unwinding of DNA by the helicase. A recent study shows that Topo III α and BLM cooperate to resolve the double-holliday junction, an intermediate produced during recombination (Wu and Hickson, 2003). Resolution of this structure could be critical to suppress the hyper-recombination and the elevated SCE frequency observed in BS. In addition, Topo III α and BLM in *Xenopus* bind interdependently to replicating chromatin, and BLM is required to prevent accumulation of DSBs during normal replication (Li *et al*, 2004). BLM also interacts with replication protein A (RPA) (Brosh *et al*, 2000), a DNA binding protein essential for replication and nucleotide excision repair; MLH1 (Langland *et al*, 2001; Pedrazzi *et al*, 2001), a protein involved in mismatch repair; and p53 (Wang *et al*, 2001), a tumor suppressor. Moreover, BLM has been found in the BRCA1-associated genome surveillance complex, which contains at least 10 proteins involved in repair or DNA damage response (Wang *et al*, 2000).

To assess independently the complement of proteins that are associated with BLM, we have immunopurified three BLM-containing complexes from HeLa nuclear extract (Meetei *et al*, 2003b). We found that BLM complex I (also named BRAFT) consists of at least five Fanconi anemia (FA) proteins, Topo III α , RPA and several novel polypeptides, which are termed BLAPs (for BLM-associated polypeptides). FA resembles BS in genomic instability and cancer predisposition. The fact that these disease proteins are present in the same complex suggests a connection between the BLM and FA pathways of genome maintenance. BLM complex II has a composition similar to complex I, but lacks FA proteins. BLM complex III contains Topo III α , MLH1 and BLAP75, but lacks both RPA and FA proteins. Here we describe characterization of BLAP75, a polypeptide common to all three BLM complexes. We show that BLAP75 is an integral component of BLM complexes, and loss of BLAP75 resulted in destabilization of BLM complexes and genomic instability.

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Results

BLAP75 is an integral component of the BLM complexes

We used sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) coupled with either silver or Coomassie blue staining to analyze BLM complexes immunopurified from HeLa cells. A 75 kDa polypeptide (BLAP75) was always present in near-equal molar amounts compared to components such as BLM and Topo III α (Meetei *et al*, 2003b) (Figure 1A and B), hinting that this polypeptide could be a stoichiometric subunit of the BLM complexes. Mass spectrometric analysis identified the polypeptide as C9orf76 (approved gene name; chromosome 9 open reading frame 76; accession ID: AK022950; alternative ID: FLJ12888), a hypothetical protein with no known function.

We also immunopurified BLM complex I (BRAFT) by using an antibody against an FA protein, FANCA (Meetei *et al*, 2003b), and found the identical 75 kDa polypeptide in this preparation (referred to as FAAP75) by mass spectrometry (MS) analysis (data not shown). The fact that the same protein can be purified by antibodies against two separate components of the complex strongly suggests that C9orf76 is an integral component of the complex and not a contaminant.

We made two polyclonal antibodies against different regions of C9orf76 and found that both antibodies recognized BLAP75 by immunoblotting (Figure 1C and data not shown), verifying that C9orf76 is BLAP75. In addition, immunopurification by BLM antibody significantly depleted BLAP75 in the flow-through fraction (Figure 1C, compare lanes 4 and 5), indicating that most of BLAP75 in cells is present within BLM complexes. For comparison, the level of RPA remains largely unchanged after immunopurification, consistent with previous findings that only a small fraction of cellular RPA is present in the BLM complexes (Meetei *et al*, 2003b).

To assess independently if BLAP75 is an integral component of BLM complexes, we used a BLAP75 antibody to immunopurify BLAP75 and its associated polypeptides. These polypeptides, when analyzed by SDS gel coupled with silver or Coomassie staining, displayed a pattern and stoichiometry nearly identical to those isolated by the BLM antibody (Figure 1A and B). MS analysis and immunoblotting identified these polypeptides as BLM, Topo III α , RPA, BLAP75 and BLAP250, all of which are components of the BLM complexes (Figure 1B and C). That the independent purification with BLAP75 antibody obtained the BLM complex components reinforces the notion that BLAP75 is an integral component of BLM complexes.

BLAP75-containing complexes are smaller in BLM-deficient cells

To further characterize the association of BLAP75 with BLM, we tested their cofractionation by Superose 6 gel filtration chromatography. The Superose 6 profiles of these two proteins in HeLa nuclear extract are highly similar as shown in Figure 1D. The major peaks representing each protein are present in the same 1–1.5 MDa fractions (17 and 18), sizes that correspond to the BRAFT complex reported previously (Meetei *et al*, 2003b).

If two proteins are components of a single complex, one would expect when one component is missing, the complex containing the other protein should fractionate at a smaller size by Superose 6 sizing column. We have previously used this assay to validate the association of several other complexes (Meetei *et al*, 2003b; Xue *et al*, 2003). Here we found that majority of BLAP75 in the extract from a BLM-deficient cell line (GM08505) fractionated in low-molecular-weight fractions, with its peak (fraction 28) corresponding to a complex of 440 kDa (Figure 1D). Importantly, when the same cell line complemented by expressing green fluorescence protein-tagged BLM (GFP-BLM) (Hu *et al*, 2001) was analyzed, BLAP75 fractionated at a relatively high molecular weight mass, with its peak (fraction 22) corresponding to a complex of about 800 kDa. Thus, the data are consistent with the notion that BLAP75 associates with GFP-BLM to form high-molecular-weight complexes in the complemented GM08505 cells.

We noticed that although the gel filtration profiles of GFP-BLM and BLAP75 from the complemented GM08505 cells overlap, the similarity between the two is not as striking as that of HeLa cells, suggesting that the assembly of the complexes containing these proteins in the complemented GM08505 cells is not as efficient as that in HeLa cells. This may be because GFP-BLM is expressed from a viral promoter (CMV), so that its expression level may not be properly regulated like that of the endogenous BLM, which is known to be tightly controlled during cell cycle progression (Dutertre *et al*, 2000); the optimal level of BLM may be necessary for its efficient association with BLAP75 into various complexes.

BLAP75 is conserved in multiple eukaryotic species and contains a putative OB-fold domain

Sequence analysis revealed that BLAP75 has sequence orthologs in many eukaryotic species, including mouse, fish (*Danio rerio* and *Ciona intestinalis*) and *Caenorhabditis*

Figure 1 BLAP75 is an integral component of BLM complexes. (A) Silver-stained and (B) Coomassie blue-stained SDS gels showing that the major polypeptides immunopurified by BLM and BLAP75 antibodies are identical, suggesting that these two proteins, together with the isolated polypeptides, are all components of BLM complexes. The major polypeptides visualized on Coomassie-stained gel (marked by arrows) from both preparations have been identified by MS. As controls, mock immunoprecipitation (IP) was carried out by using either a preimmune serum or protein A beads alone. The affinity-purified BLAP75 antibody was also shown as a control. The pattern of BLM-associated polypeptides reported here is somewhat different from the previous study (Meetei *et al*, 2003b), because preparation of the nuclear extracts used in these studies is different (see Materials and methods). It is noticed that the amount of antigen subunit (e.g., BLM in BLM IP) is lower compared to other components of the complex in (A). The reason could be that the antigen subunit binds directly to the polyclonal antibody beads, so that it is eluted less efficiently than other components of the complex under this condition. (C) Immunoblotting to show that BLAP75, BLM, Topo III α and RPA are present in complexes immunopurified by both BLM and BLAP75 antibodies, but absent in mock purifications using preimmune serum. The analysis was performed for the load, flow-through (FT) and the immunoprecipitated (IP) fractions. (D) Immunoblotting to show the Superose 6 gel filtration profiles of BLM and BLAP75 in nuclear extracts prepared from HeLa cells (upper two panels), a BS patient cell line (GM08505) (middle two panels) and the same patient cell line complemented by stably expressing GFP-BLM (bottom two panels). Notably, majority of BLAP75 in HeLa and GFP-BLM complemented cells fractionate in high-molecular-weight fractions (> 670 kDa), but fractionate in low-molecular-weight fractions (about 440 kDa, fraction 28) in the BS cell line, suggesting that in the absence of BLM, the BLAP75 complex becomes smaller.

elegans, although there were no obvious orthologs in *Drosophila* or yeast (Figure 2A). A genome-wide siRNA knockdown study in *C. elegans* found that depletion of the worm ortholog of BLAP75 (T07C12.12) resulted in embryonic

lethality in about 10% of embryos, suggesting that it may be essential for embryonic development (Piano *et al*, 2002).

BLAP75 was annotated as 'nucleic acid binding' by the Gene Ontology Annotation (GOA) project of European

Figure 2 BLAP75 contains a putative OB-fold nucleic acid binding domain and is conserved in multiple eukaryotic species. (A) Sequence alignment of BLAP75 family of proteins from multiple eukaryotic species. The conserved N- and C-terminal regions are marked by brackets. The predicted β -sheets for the OB-fold nucleic acid binding domain are underlined. The abbreviations and accession numbers for different BLAP75 orthologs are as follows: human (humBLAP75; also called C9orf76 or FLJ12888; NP_079221), mouse (mouBLAP75; NP_083180.2), *C. intestinalis* (cioBLAP75; AK114784), *D. rerio* (danBLAP75; AAH45482.1) and *C. elegans* (celBLAP75; NP_741607.1). (B) Sequence alignment to show that part of the N-terminal domain of BLAP75 is also conserved in the Tudor domain 3 (Tdrd3) family of proteins. The abbreviations and accession numbers of these proteins are as follows: human (humTdrd3; also called FLJ21007; NP_110421.1), *D. rerio* (danTdrd3; AAH55609.1), *Xenopus tropicalis* (xenTdrd3; NP_989385.1), *Drosophila* (droTdrd3; NP_648724.1) and *C. elegans* (celTdrd3; NP_503358.1). A hypothetical protein of *Schizosaccharomyces pombe* (Yau3_schpo; NP_594146.1) was also found to contain the domain and was included in the alignment, even though it is not a member of the Tdrd3 family.

Bioinformatics Institute, based on the presence of an OB-fold domain, which is a structural motif frequently used in nucleic acid recognition (Theobald *et al*, 2003). Several proteins containing this domain, including RPA and BRCA2, participate in DNA replication or repair.

Examination of BLAP75 and its orthologs revealed the presence of two conserved domains at each terminus of the protein. Searching the Protein Families Database (pfam)

revealed that the N-terminal domain has weak homology to the OB-fold domain.

The N-terminal domain of BLAP75 also contained homology with the N-terminal region of another family of proteins, termed TDRD3 for Tudor domain-containing protein 3 (Figure 2B). The TDRD3 proteins have no known functions, but they all contain a conserved Tudor domain, which has recently shown to bind methylated lysine 79 of histone H3

and to target 53BP1 (a checkpoint protein) to DNA double-strand breaks (Huyen *et al*, 2004). These proteins also contain a ubiquitin-associated domain, which can interact with ubiquitin and has been found in proteins involved in ubiquitination, DNA repair and intracellular signaling (Buchberger, 2002). The fact that this N-terminal domain is present in two distinct families of proteins suggests that it may have a broad functional role that has been conserved throughout evolution.

BLAP75 is essential for stability of BLM complexes

When BLAP75 was depleted in HeLa cells by siRNA, a drastic decrease in Topo III α protein level (10-fold or more) and a moderate reduction in BLM protein level (about two- to three-fold) was observed (Figure 3A). In the reciprocal experiment, depleting BLM by siRNA has no significant effect on the BLAP75 protein level (Figure 3B). In a BLM-deficient cell line derived from a BS patient, the BLAP75 level was also comparable to that of cells from a normal individual. Thus, whereas BLAP75 is required for stability of BLM and Topo III α , BLM is not required for stability of BLAP75. We have previously shown that the level of Topo III α in BLM-deficient cells is also comparable to that of cells from a normal individual (Meetei *et al*, 2003b), suggesting that the stability of Topo III α is specifically dependent on the presence of BLAP75, but not BLM, even though all three proteins are components of the same complexes. For many multiprotein complexes, absence of one subunit could render others unstable (Peterson and Herskowitz, 1992; Meetei *et al*, 2004). The finding that BLAP75 is required for stability of BLM and Topo III α once again confirms the notion that BLAP75 is a critical and integral component of BLM complexes.

BLM is known to be cleaved by caspase 3 during apoptosis, generating two major fragments of 110 and 47 kDa (Bischof *et al*, 2001a; Freire *et al*, 2001). It is therefore possible that the reduced levels of BLM and Topo III α proteins in BLAP75-depleted cells may be a side effect of increased apoptosis. The following lines of evidence are against this possibility. First, the level of 110 kDa BLM cleavage product is strongly increased when HeLa cells were induced to undergo apoptosis by treatment of TNF α and cycloheximide (Figure 3C). However, this product remains almost undetectable in BLAP75-depleted HeLa cells, indicating that the degradation of BLM in these cells is not by caspase 3. Second, a highly sensitive apoptosis marker—the cleavage of poly (ADP-ribose) polymerase (PARP)—was not observed in BLAP75-depleted cells, indicating absence of significant apoptosis by BLAP75 siRNA treatment. Third, flow cytometry revealed that the cell cycle progression of BLAP75-depleted cells is comparable to those treated with control oligos (Figure 3D). In particular, the level of sub-G1 cells, which have less than 2N DNA and have often been used as an indicator for apoptotic cells (see Figure 3D, bottom panel as a positive control), remains at the background level (less than 1% of total cells) for BLAP75-depleted cells (Figure 3D). Moreover, analysis of cell viability by Trypan blue staining showed that for cells treated with either BLAP75 siRNA or control oligos, over 99% of cells are viable (data not shown), again suggesting no significant increase in cell death. Fourth, the reduced level of Topo III α was observed only in BLAP75-depleted cells, but not in cells undergoing apoptosis (Figure 3C). These data clearly demonstrate that BLAP75 depletion does not lead

to increased apoptosis, and its effect on the level of Topo III α and BLM should be specific.

BLAP75 is essential for phosphorylation of BLM during mitosis

BLM becomes hyperphosphorylated when cells are blocked at mitosis by chemicals such as Taxol or demecolcine (Dutertre *et al*, 2000; Beamish *et al*, 2002). Hyperphosphorylated BLM can be easily distinguished from the hypophosphorylated form based on their differential mobility on SDS-PAGE (Figure 3E). Notably, when HeLa cells depleted of BLAP75 were blocked at mitosis, little or no hyperphosphorylated BLM was detected, indicating that BLAP75 is required for BLM phosphorylation during mitosis.

BLAP75 is required for normal cell proliferation

We noticed that HeLa cells treated with BLAP75 siRNA oligos appeared to have a lower cell density than those treated with a control oligo. Analysis by Trypan blue staining and manual counting revealed that the number of viable cells after treatment of BLAP75 siRNA is about 90% of that of the control siRNA-treated cells 48 h post-transfection, and about 75% of the control cells after 72 h (Figure 4A). This reduced cell number is not due to increased cell death, because the number of dead cells (stained positive by Trypan blue) is very low, representing less than 1% of total cells (data not shown). Data from flow cytometry and biochemical analysis of apoptosis markers also excluded apoptosis as the cause for this reduction (Figure 3). One possible cause could be reduced proliferation in BLAP75-depleted cells.

In order to monitor proliferation of cells for a prolonged period, we generated lentivirus-based siRNA vectors to stably knock down BLAP75 in HeLa cells (Figure 4B). The HeLa cells infected with a BLAP75 siRNA vector displayed drastically reduced efficiency in colony formation compared to cells infected with a control lentivirus vector (Figure 4C). A similar result was also obtained by transfection of HCT116 cells with a plasmid vector expressing a different BLAP75 siRNA (Figure 4D and E). Thus, BLAP75 may be essential for sustained cell proliferation.

BLAP75 resembles BLM in localizing to nuclear foci in response to DNA damage

In response to DNA damage, BLM and several other proteins involved in DNA repair, replication and genome surveillance form large nuclear foci, which are subnuclear structures that can be visualized microscopically as bright nuclear dots (Wang *et al*, 2000; Bischof *et al*, 2001b; Wu *et al*, 2001). These foci have been observed in close proximity to DNA damage sites (Davalos and Campisi, 2003), and may play important roles in DNA repair. By indirect immunofluorescence, we found that BLAP75 displays a diffused nuclear staining pattern in the majority of HeLa cells, and forms foci only in a small percentage of cells (Figure 5A and D). However, when cells were treated with agents that either induce DNA damage or block replication, such as ionizing radiation (IR), mitomycin C (MMC), hydroxyurea, aphidicolin and diepoxybutane (DEB), the percentage of cells containing BLAP75 foci is drastically increased (about 10-fold) (Figure 5A and B and data not shown; see Figure 5D for quantitation). This increase is comparable to the induction of BLM foci under identical experimental conditions (Figure 5D). The fact that BLAP75

forms foci in response to different DNA damage agents supports its requirement for DNA repair pathways.

BLAP75 colocalizes with BLM and is essential for BLM foci formation

The biochemical data demonstrate that BLAP75 and BLM are components of the same complexes. In accord with such data, immunofluorescence analyses found that in HeLa cells

treated with MMC, the BLAP75 foci almost completely overlapped with those of BLM (Figure 5B). Moreover, in cells depleted of BLAP75 by siRNA, the number of MMC-induced BLM foci is reduced to the level observed in cells that were not exposed to MMC (compare Figure 5C and B; also see Figure 5D for quantitation). Once again, we infer that BLAP75 is a critical component of BLM complexes that is essential for the recruitment of BLM to foci in response to DNA damage.

BLAP75-depleted cells have an elevated frequency of SCE

The hallmark feature of BLM-deficient cells is their elevated frequency of SCE. To determine if cells deficient in BLAP75

Figure 4 BLAP75 is required for sustained cell proliferation. (A) Histogram to show that BLAP75 depletion reduces the total number of viable cells when compared to the cells treated with a control oligo. The viable cells are distinguished from dead ones by Trypan blue staining. (B) Immunoblotting to show that HeLa cells infected with BLAP75 siRNA in lentivirus vector have reduced expression of BLAP75, in comparison to same cells infected with a control siRNA using the same vector. The quantitated results (shown at the bottom) were obtained by scanning with a Typhoon Scanner and ImageQuant, and the normalization was performed using actin as a control. (C) Histogram to show that the number of colonies (which reflect sustained cell proliferation) is drastically reduced for cells treated with BLAP75 siRNA vector compared to those with the control vector. (D, E) Similar data as (B, C) except that a different BLAP75 siRNA vector was used, and the experiment was performed in HCT116 cells.

expression have elevated SCE, we used an siRNA-based strategy to deplete BLAP75. The SCE level in BLAP75-depleted HeLa cells was elevated, similar to that observed for BLM-depleted HeLa cells ($P < 0.001$) (Figure 6). We infer that BLAP75 and BLM have similar roles in maintaining genome integrity by suppressing hyper-recombination and SCE. The level of SCE observed in HeLa cells for both BLM and BLAP75 depletion is lower than that of BLM patient cells, suggesting that siRNA technology cannot completely mimic BS patient cells in which BLM is completely absent.

Discussion

We have previously demonstrated that in HeLa nuclear extract, BLM always exists in multiprotein complexes (Meetei *et al*, 2003b). We have purified three such complexes and shown that one of their common components is Topo III α , a protein essential for BLM function. Here we showed that another common component of these complexes, BLAP75, is required for BLM complex stability, BLM mitotic phosphorylation and recruitment of BLM to nuclear foci in response to DNA damage. Moreover, depletion of BLAP75 results in an elevated frequency of SCE, a characteristic feature of BLM-deficient cells. Thus, our data not only identified BLAP75 as a new component of the BLM complexes, but also implicate BLAP75 as an essential partner of BLM in maintaining genome integrity. Among the large number of proteins that are found as components in purified BLM complexes, or shown to interact with BLM by other means, BLAP75 is one of only two proteins that always copurify with BLM (the other one is Topo III α). Thus, we propose that BLAP75, together with Topo III α , forms the centerpiece for BLM function.

Because BLM, Topo III α and BLAP75 are present in near-stoichiometric amounts in all three major BLM complexes purified from HeLa cells, we propose that these proteins work as a unit and constitute a structural core for various BLM complexes. The findings that BLAP75 and BLM cofractionate and copurify with each other (Figure 1), and that Topo III α is nearly completely degraded in BLAP75-depleted cells (Figure 3A) suggest that these three proteins may work exclusively as parts of this core and are indispensable for each other's function. Other BLM complex components could be considered as auxiliary subunits, which can assemble with this core to produce multiple complexes, thus adapting the

Figure 3 BLAP75 is required for BLM complex stability and mitotic phosphorylation of BLM. (A) Immunoblotting to show that suppression of BLAP75 expression by siRNA oligos reduces the levels of BLM and Topo III α in HeLa cells. As control, an siRNA oligo with scrambled sequence fails to reduce BLAP75 expression as well as the levels of BLM and Topo III α . Immunoblotting of β -actin is shown as loading control. (B) Immunoblotting to show that the level of BLAP75 is not significantly altered in cells depleted of BLM by siRNA, or in a BS patient cell line (BLM $^{-/-}$) that lacks BLM. A lymphoblastoid cell line derived from a normal individual (WT) was used as a control. (C) Immunoblotting to show that the reduction of Topo III α and BLM protein levels in BLAP75-depleted HeLa cells is not due to increased apoptosis. HeLa cells were either untreated or treated with apoptosis-inducing drugs, TNF α and cycloheximide, as shown at the top of the figure. The presence of the specific cleavage product of PARP (Δ PARP) is a marker for cells undergoing apoptosis. Notably, the appearance of caspase 3 cleavage product of BLM (Δ BLM) correlates with apoptosis induction, but not BLAP75 depletion. In contrast, reduction in Topo III α protein level correlates with BLAP75 depletion, but not apoptosis induction. Polypeptides that crossreact with BLAP75 antibodies are marked by asterisks. (D) Flow cytometry histograms to show normal cell cycle progression and absence of apoptosis for BLAP75-depleted cells. The percentages of cells at different stages of cell cycles are shown within figures for HeLa cells growing under normal conditions (upper panel). Notably, the proportion of sub-G1 cells (marked by a bracket with $< G1$), which have less than 2N DNA content and include apoptotic cells, is very low under normal growth conditions (less than 1%), but is significantly increased when cells undergo apoptosis (bottom panel). (E) Immunoblotting to show that suppression of BLAP75 expression by siRNA reduces mitotic phosphorylation of BLM. Cells were treated without any drugs, or with mitotic inhibitors, Taxol and demecolcine, as indicated. The cells at different stages of the cell cycle were determined by flow cytometry analysis, and the percentages of cells at G2 and M phases are shown at the bottom. The hyper- and hypo phosphorylated BLM proteins are marked by a solid and an empty dot, respectively. A possible degradation product of BLM is marked by an asterisk.

core to multiple cellular functions, including maintenance of genomic stability. Among these auxiliary subunits, RPA and BLAP250 were isolated as major polypeptides by independent

purifications using antibodies against either BLM or BLAP75 (Figure 1B), suggesting that they may also play a major role in mediating the function of this core.

We have previously shown that one of the BLM complexes, BRAFT, contains at least six FA core proteins in addition to the BLM core proteins (Meetei *et al*, 2003a,b). Consistent with this association, BLAP75 was found to be identical to FAAP75, a polypeptide isolated by immunopurification using an antibody against an FA protein, FANCA. This raised the possibility that BLAP75 may also be an FA core protein participating in the FA DNA damage response pathway. However, using the FANCD2 monoubiquitination assay (which is a convenient way to determine whether a protein is a functional FA core complex protein), we found that depletion of BLAP75 by siRNA has no significant effect on the level of monoubiquitinated FANCD2 (data not shown). This is in contrast to depletion of FA core proteins, which results in drastic reduction of monoubiquitinated FANCD2 (Bruun *et al*, 2003; Meetei *et al*, 2003a). Moreover, BLAP75-depleted cells have defective mitotic phosphorylation of BLM, whereas FANCA-deficient cells do not (data not shown). Together, these data argue that BLAP75 is not directly required for function of the FA pathway.

Although BLAP75 is an essential part of the BLM–Topo III α core complex, its exact roles remain obscure. It might have

a purely structural role, allowing proper assembly of the BLM and Topo III α into a stable complex. Failure in assembly of this complex apparently leads to degradation and subsequent reduction of Topo III α and BLM proteins (Figure 3). Because Topo III α is necessary for maintaining genome stability by BLM, the drastic reduction in its level may account for the increased level of SCE in these cells. Although BLAP75 is required for phosphorylation of BLM in mitosis, we failed to find any kinase domains within the BLAP75 sequence, implying that its role in BLM phosphorylation could be indirect. A recent study has shown that phosphorylation of *Xenopus* homolog of BLM depends on the presence of Topo III α (Li *et al*, 2004). Thus, depletion of BLAP75 may indirectly affect BLM phosphorylation through destabilization of Topo III α . Another potential function for BLAP75 could be DNA binding, based on the presence of its putative OB-fold nucleic acid binding domain. However, we have been unable to show that this domain has any DNA binding activity *in vitro* using recombinant proteins (data not shown). Thus, the contribution of this domain to DNA binding in the context of the entire complex remains to be investigated.

Based on the strong biochemical and functional connections between BLAP75 and BLM, one might predict that mutations in the BLAP75 gene may cause disease phenotypes similar to BS, including cancer predisposition. On the other hand, several lines of evidence hint that individuals with null mutations in BLAP75 might not survive. Stable depletion of BLAP75 resulted in poor proliferation of HeLa and HCT116 cells, and depletion of the *C. elegans* ortholog of BLAP75 by siRNA caused partial embryonic lethality (Piano *et al*, 2002). Also, complete absence of BLAP75 could indirectly cause embryonic lethality through destabilization of Topo III α , which has been shown to be essential in mouse embryonic development (Li and Wang, 1998). Nevertheless, certain mutations that do not cause instability of Topo III α might allow individuals to survive and develop diseases. In particular, BLAP75 is located at chromosome 9q22.1, a region reportedly with frequent loss of heterozygosity in Chinese esophageal squamous cell carcinomas (Lichun *et al*, 2004). It remains to be seen whether BLAP75 is mutated in this or other cancers.

Materials and methods

Chemicals

Demecolcine and Taxol (Sigma) were resuspended in DMSO to a stock concentration of 0.26 and 10 mM, respectively, and used at the respective dilutions 1:1000 and 1:10 000. Aphidicolin and hydroxyurea (Sigma) were resuspended in DMSO and water, respectively, to a stock concentration of 1 mg/ml and 1 M, respectively, and used at the respective dilutions 1:2000 and 1:1000. TNF α and cycloheximide (Sigma) were used at 10 ng/ml and 20 μ g/ml final concentration, respectively.

Figure 6 BLAP75-depleted cells display increased frequency of SCE similar to BLM-depleted cells. A histogram to show that depletion of BLAP75 by siRNA results in elevated frequency of SCE (as defined by the number of SCE per chromosome). HeLa cells treated with a scrambled oligo or a BLM siRNA oligo were used as negative and positive controls. SV40-transformed fibroblast cell lines from a normal individual (BLM+/+) and a BS patient (BLM-/-) are also shown for comparison. More than 600 chromosomes were scored for each group. Error bars represent standard deviation of each sample. Statistical analyses of the SCE data were performed using both one-factor ANOVA (analysis of variance) and the Student's two-tailed *t*-test. The results show that differences between control cells and those depleted of either BLM or BLAP75 are statistically significant, and the *P*-values (<0.001) were included in the figure.

Figure 5 BLAP75 colocalizes with BLM in nuclear foci in response to DNA damage and is required for BLM foci formation. (A) Images from indirect immunofluorescence analysis to show that BLAP75 in HeLa cells forms nuclear foci in response to several DNA damage reagents. DNA was visualized by costaining with Hoechst dye. (B) Representative immunofluorescence images to show that BLAP75 and BLM colocalize in nuclear foci in response to the DNA crosslinking drug MMC (50 ng/ml). These cells were also treated with a scrambled siRNA oligo to serve as a control for the next figure. (C) Representative images to show that HeLa cells treated with BLAP75 siRNA oligo display not only reduced BLAP75 staining but also reduced number of BLM foci. (D) Histogram of quantitative analysis of cells containing BLAP75 and BLM foci to illustrate that the percentage of cells containing BLM foci is drastically reduced in HeLa cells depleted of BLAP75 by siRNA oligo. A scrambled siRNA oligo was transfected in cells that were untreated with BLAP75 siRNA oligo to serve as a control (marked as 'BLAP75 siRNA -'). The presence or absence of MMC is also indicated. Nuclei were tabulated in groups containing 0–5 and >5 foci per nucleus.

Antibodies

Two rabbit BLAP75 polyclonal antisera were raised against fusion proteins containing maltose binding protein (MBP) and two regions of BLAP75 (residues 183–262 and 286–336, respectively). All polyclonal antisera were affinity purified and used for immunoprecipitation and immunoblotting. A polyclonal antibody against BLM (69D) has been described elsewhere (Meetei *et al*, 2003b), and the BLM antibody (ab476) was from Abcam. A Topo III α antibody was kindly provided by Dr B Johnson. A mouse monoclonal BLM antibody was a gift of Dr P Moens. Mouse monoclonal antibodies to RPA32 (AB1) and RPA70 (AB2) were from Neomarker. A mouse PARP antibody (551024) was from BD Pharmingen. A monoclonal anti- β -actin antibody (AC-15) was from Sigma-Aldrich.

Cell culture

Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines, one derived from a normal individual (ManEBV) and another from a BS patient (2036), were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. The cells were grown in a humidified 5% carbon dioxide (CO₂)-containing atmosphere at 37°C. SV40-transformed fibroblast cell lines, one derived from a BS patient (GM08505) and one from a normal individual (GM00637), were obtained from the Coriell Cell Repositories. The BS patient cell line (GM08505) and its derivative complemented by GFP-BLM were from Dr N Ellis (Hu *et al*, 2001). These cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. HeLa S3 cells were obtained from the National Cell Culture Center. For MMC-treated HeLa cells, the cells were treated with 40 ng of MMC/ml for 24 h. For cell cycle analysis, cells were treated with propidium iodide (50 μ g/ml) and RNase (1 mg/ml) for 1 h at room temperature, and analyzed by flow cytometry (FACScan, BD Immunocytometry Systems). The cell cycle percentages were analyzed using multiple analysis software (Phonexix Flow, San Diego, CA).

Fractionation

Nuclear and cytoplasmic extracts were prepared essentially as described (Dignam *et al*, 1983), except for one important difference. Briefly, the nuclear extract was usually prepared by extracting nuclear pellet twice using buffer C (20 mM HEPES pH 7.9, 0.42 M NaCl, 25% glycerol, 1.5 mM MgCl₂ and 0.2 mM EDTA). The final salt concentration of the first extract is about 0.25 M, whereas that of the second extract is about 0.35 M. Proteins are differentially extracted due to the different salt concentrations of the extraction buffers. The first extract, which was used in Figure 1A and B of the present study, has a higher level of BLM complex II (that lacks FA and MLH1). Conversely, the second extract, used in the previous study (Meetei *et al*, 2003b), has a higher proportion of the BLM complex I (containing FA proteins) and BLM complex III (containing MLH1). Thus, the complex reported in Figure 1 of this study is mainly BLM complex II (although we can still detect FANCA and MLH1 by Western in this preparation), whereas the complexes described previously have all three complexes in roughly equal amounts.

The nuclear extract was directly applied to a Superose 6 column (HR16/50; Amersham Pharmacia Biotechnology) equilibrated with the column running buffer containing 20 mM HEPES (pH 7.9), 200 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μ g/ml leupeptin, 2 μ g/ml aprotinin, 0.1% NP-40 and 5% glycerol. Fractions were collected and analyzed by SDS-PAGE and immunoblotting.

Immunoprecipitation

BLM and BLAP75 complexes were isolated from HeLa S3 nuclear extracts by using a modified immunoprecipitation protocol described previously (Lee *et al*, unpublished data; Meetei *et al*, 2003b). Briefly, nuclear extracts were incubated with rabbit polyclonal anti-BLM or anti-BLAP75 antibodies, respectively, in the presence of 100 μ l of protein A beads (Amersham Pharmacia) for at least 12 h at 4°C. The immunoprecipitates were washed four times with the IP buffer (20 mM HEPES (pH 7.9), 200 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 2 μ g/ml leupeptin and 2 μ g/ml aprotinin). The complex was eluted from the beads by using 100 mM glycine-HCl buffer (pH 2.5). The eluted complex was subjected to SDS-PAGE and immunoblotting analysis. Methods for MS have been described elsewhere (Meetei *et al*, 2003b).

siRNA experiment

siRNA duplexes (21 nt) with 3'dTdT overhangs corresponding to BLAP75 mRNA (oligo1: AGCCUUCACGAAUGUUGAU; oligo2: UCUAGUUACAGCUGAAGCA) and BLM mRNA (oligo1: AGCAGC GAUGUGAUUUGCA; oligo2: AUCAGCUAGAGCGCAUCAA) were synthesized (Dharmacon). The sequence AUUGAUGCGAUCGCA GAC was used as a nonspecific RNAi control. HeLa cells were transfected with siRNA or control oligos using Oligofectamine following the manufacturer's instructions (Invitrogen). After 48 h, the cells were seeded either on chamber slides (for microscopic analysis) or in tissue culture plates, and incubated for another 24 h with or without DNA damage agents or cell cycle-blocking drugs. Apoptosis was induced by incubating cells in the presence of 10 ng/ml TNF α and 20 μ g/ml cycloheximide for 6 h.

For clonogenic survival assay, the following BLAP75 siRNA sequences were used: siRNA 1: ATCTAGTTACAGCTGAAGC; siRNA 2: GAGCAGTGGCTCCTTACTGAT. HeLa cells were infected by lentiviruses containing BLAP75 or control (luciferase) siRNA expression cassette (*Proc Natl Acad Sci* (2003); **100**: 183–188). After 24 h, cells were replated at low density and allowed 10–12 days for colony formation. Fixed cells were stained with crystal violet and the visible colonies (> 50 cells) were counted. For plasmid-based siRNA delivery, BLAP75 or control siRNA was expressed from the pDsU6 vector (Bao *et al*, 2001, 2004). The siRNA constructs were transfected with FuGENE 6 (Roche) followed by a 3-day post-transfection selection with G418 (400 μ g/ml). The cells were then plated at low density in the absence of G418 to allow colony formation.

SCE assay

SCE staining was performed as described (Perry and Wolff, 1974). At 36 h after HeLa cells were transfected with siRNA oligos, cultures were grown through two cell cycles to achieve preferential labeling of sister chromatid in the presence of 100 μ M of 5-bromodeoxyuridine. Colcemid (Sigma) was added at a final concentration of 0.05 μ g/ml to accumulate mitotic cells 2 h prior to harvesting cells. Harvested cells were then incubated in hypotonic solution (0.06 M KCl) for 15 min at room temperature, and then fixed with 3:1 (vol/vol) methanol-glacial acetic acid. Fixed cell suspension was dropped onto a glass slide and air-dried. Sister chromatid differentiation was performed by the fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). More than 600 chromosomes were scored for each cell group treated with BLAP75, BLM or control siRNA oligo. Two SV40-transformed fibroblast cell lines, one derived from a BS patient (GM08505) and one from a normal individual (GM00637), were analyzed similarly. Statistically analyses of the SCE data were performed using both one-factor ANOVA (analysis of variance) and the Student's two-tailed *t*-test.

Immunofluorescence

Indirect immunofluorescence was carried out as described (Meetei *et al*, 2003a). HeLa cells grown on chamber slides were washed with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde, permeabilized with PBS containing 0.2% Triton X-100 and blocked with a solution of 7.5% BSA in PBS. Primary and secondary antibodies were diluted in PBS containing 0.5% BSA. Cells were incubated in primary antibody (1:3000 for anti-BLAP75 (Rabbit), 1:400 for anti-BLM (mouse)) for 1 h at 4°C. Following five 5-min washes with PBS, secondary antibody was added at 1:1000 (Alexa Fluor 594-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse; Molecular Probes). Nuclei were counterstained with Hoechst 33342 dye (Molecular Probes). The cells were mounted with anti-fade mounting solution (Vectashield, Vector Laboratories). For statistical analysis of nuclear foci formation, wide-field images were taken using a Nikon TE800 microscope with an Optronics DEI750 camera. Images were processed using Adobe Photoshop 7.0. To analyze colocalization of two proteins in nuclear foci, deconvolution images were acquired with the Applied Precision 'Image Restoration System' with an inverted Nikon Eclipse TE200 microscope with standard filter sets, and a CH350L camera and Delta Vision software. Quantification of nuclear foci was carried out by capturing high-field images containing up to 5–20 nuclei. Single nuclei were scored for BLAP75 foci and BLM foci. Statistical evaluation of foci-containing nuclei was performed using Microsoft Excel.

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