DNA Damage and Cellular Stress Responses

Chk1-Dependent Constitutive Phosphorylation of BLM Helicase at Serine 646 Decreases after DNA Damage

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Abstract

BLM helicase, the protein mutated in Bloom syndrome, is involved in signal transduction cascades after DNA damage. BLM is phosphorylated on multiple residues by different kinases either after stress induction or during mitosis. Here, we have provided evidence that both Chk1 and Chk2 phosphorylated the NH2-terminal 660 amino acids of BLM. An internal region within the DExH motif of BLM negatively regulated the Chk1/Chk2-dependent NH2-terminal phosphorylation event. Using in silico analysis involving the Chk1 structure and its known substrate specificity, we predicted that Chk1 should preferentially phosphorylate BLM on serine 646 (Ser646). The prediction was validated in vitro by phosphopeptide analysis on BLM mutants and in vivo by usage of a newly generated phosphospecific polyclonal antibody. We showed that the phosphorylation at Ser646 on BLM was constitutive and decreased rapidly after exposure to DNA damage. This resulted in the diminished interaction of BLM with nucleolin and PML isoforms, and consequently decreased BLM accumulation in the nucleolus and PML nuclear bodies. Instead, BLM relocalized to the sites of DNA damage and bound with the damage sensor protein, Nbs1. Mutant analysis confirmed that the binding to nucleolin and PML isoforms required Ser646 phosphorylation. These results indicated that Chk1-mediated phosphorylation on BLM at Ser646 might be a determinant for regulating subnuclear localization and could act as a marker for the activation status of BLM in response to DNA damage.

Introduction

Signal transduction during DNA damage response is mediated via two proximal sensory kinases, ATM (ataxia-telangiectasia–mutated) and ATR (ATM-Rad3-related; refs. 1, 2). ATR and ATM initiate the signaling cascade via phosphorylation of its downstream checkpoint effector kinases, Chk1 and Chk2 (3). Although ATR/Chk1 predominantly sensed the damage in response to stalled replication, ATM/Chk2 were involved in response to double-strand breaks. ATM/ATR along with Chk1/Chk2 are known to phosphorylate a variety of downstream targets involved in different cellular process including DNA damage response.

The highly conserved family of proteins, RecQ helicases, is involved in DNA damage response in humans (4, 5). Mutation in three members of the RecQ helicase family led to cancer predisposition syndromes: Bloom syndrome (due to mutation in BLM), Werner syndrome (due to mutation in WRN), and Rothmund-Thomson syndrome (due to mutation in RTS). Patients with Bloom syndrome (BS) are prone to almost all forms of cancer, thereby possibly indicating that BLM is involved in the regulation of key DNA-dependent processes such as DNA replication, recombination, and repair (6). BLM is also an upstream sensory protein involved in the DNA damage signaling cascade especially after stalled replication (7-9). Hence, it could be hypothesized that phosphorylation of BLM by these kinases would be a prerequisite for the helicase to function effectively as a DNA damage sensory protein in vivo.

ATM is the first kinase shown to be involved in the phosphorylation of BLM (10). ATM phosphorylated BLM on Thr99 and Thr122 in response to ionizing radiation, with Thr99 being the major site of phosphorylation. ATM-dependent phosphorylation on these two sites was responsible for the correction of radiation-induced damage in BS cells but had no role during sister chromatid exchange (SCE; ref. 11). BLM and ATR colocalized in the nucleus and the percentage of colocalization increased following stalling of the replication forks. ATR also phosphorylated BLM on Thr99 and Thr122 in response to hydroxyurea (HU; ref. 7). Loss of phosphorylation in Thr99/Thr122 of BLM led to a failure to recover from HU-induced replication blockade,
leading to a subsequent arrest in the caffeine-sensitive G2-M checkpoint. Phosphorylation at Thr299 was required for the colocalization of BLM with γ-H2AX (12) and 53BP1 (13). We have earlier reported that Chk1 phosphorylated BLM (8). Functional interaction of Chk1 also exists with Dna2, a known helicase cum nuclease, which could (like BLM) be recruited to the sites of replication origin and interact with sensory proteins involved in DNA damage response (like ATM and MRN; ref. 14). Chk1 (and Chk2) induction and phosphorylation occurred even in the absence of Dna2, indicating that the latter is not required for the induction or signaling of checkpoints.

Apart from the kinases activated after DNA damage (like ATM and ATR), cell cycle–specific kinases are also known to use BLM as a substrate. MPS1-dependent phosphorylation of BLM at Ser144 was required to prevent early mitotic exit and resulted in the binding of polo-like kinase 1 (PLK1) and subsequent phosphorylation of BLM (15). Phosphorylation of BLM by Cdc2 at Ser714 and Thr766 resulted in its exclusion from the chromatin and nuclear scaffold, thereby preventing BLM from interfering with mitotic processes such as chromosome condensation (16). Thus, the studies to date have indicated that BLM phosphorylation occurred either only during mitosis or in response to exogenous stress.

However, it is also possible that in addition to stimulus-induced phosphorylation, BLM could also be constitutively phosphorylated. Evidence does exist in literature regarding the constitutive phosphorylation of proteins involved in signal transduction. For example, a Chk1-mediated constitutive phosphorylation event had been observed for Cdc25B, one of the three human phosphatases that activate the CDK-cyclin complexes. Chk1 phosphorylated Cdc25B in vitro and in vivo on multiple residues, including Ser230 and Ser563. Chk1-dependent Ser230 phosphorylation was constitutively observed in the absence of DNA damage. In vivo, the mutation of Ser230 increased the mitosis-inducing activity of CDC25B, leading to the speculation that Chk1 constitutively phosphorylated Cdc25B during interphase and thus prevented the premature initiation of mitosis by negatively regulating the activity of Cdc25B at the centrosome (17). Chk1 could phosphorylate its substrates constitutively because its essential function during the cell cycle could be uncoupled from DNA damage response function and checkpoint control (18).

In this study, we wanted to determine the regulatory mechanisms governing Chk1/Chk2-mediated phosphorylations. We found that an internal region within the DEXH motif, which until now had been thought to play a role in the helicase function of BLM, could also negatively regulate NH2-terminal phosphorylation on the helicase by Chk1/Chk2. Using a structure-based in silico approach, we predicted the sites where Chk1 could phosphorylate BLM. Ser646 was predicted to be the site that would be most preferentially phosphorylated on BLM by Chk1. Using biochemical and cell biology techniques involving newly generated phosphospecific polyclonal antibody, we found that phosphorylation of BLM by Chk1 indeed occurred in vivo at Ser646. This phosphorylation on BLM by Chk1 was constitutive in nature and was diminished upon exposure to multiple types of DNA damage. Loss of Chk1-dependent Ser646 phosphorylation resulted in decreased BLM binding to nucleolin and PML isoforms, reduced accumulation in nucleolus and PML nuclear bodies (PML NB), and correlated with its relocalization (i.e., BLM) to the sites of DNA damage and binding with damage sensor protein, Nbs1. These results indicated that Ser646 phosphorylation on BLM may be one of the determinants that regulates subnuclear localization and thereby acts as a marker reflecting the activity status of the helicase.

Materials and Methods

Antibodies

A polyclonal antibody against phosphorylated Ser646 in BLM was raised in rabbits (Abexome Biosciences). Crude serum from inoculated rabbits was double–affinity-purified using phosphorylated peptide and nonphosphorylated peptide–conjugated Sepharose columns and measured for antibody concentration using an ELISA. Anti-BLM: rabbit polyclonal A300-110A (Bethyl) for Western blotting; goat polyclonal A-300-120A (Bethyl) for immunoprecipitations and immunofluorescence, anti-hsp90: sc-7947 (Santa Cruz Biotechnology), anti-nucleolin (C23): sc-8031 (Santa Cruz Biotechnology), anti-PML: sc-966 (Santa Cruz Biotechnology), anti-Nbs1: NB100-143 (Novus Biologicals), anti-geminin A/C: 612163 (BD Biosciences). Anti-Flag antibody and beads: F1804, A2220 (Sigma). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Recombinants

pGEX4T-1 BLM (1-212), pcDNA3 Flag BLM (donated by Ian Hickson), pHook Chk1 (wild-type; donated by Carol Prives), GST Chk1 (wild-type) and GST Chk1 (D130A, kinase dead mutant; donated by Stephen Elledge), pCDZF Chk2 and GST Chk2 (donated by Thanos Halazonetis). pGEX4T-1 BLM (191-660), pGEX4T-1 BLM (621-1041), and pGEX4T-1 BLM (1001-1417; all ref. 13). pGEX4T-1 BLM (191-660), pGEX4T-1 BLM (230-660), pGEX4T-1 BLM (260-320), pGEX4T-1 BLM (321-530), and pGEX4T-1 BLM (531-660) were obtained by cloning the respective PCR products into the pGEX4T-1 vector and checking the orientation. pGEX4T-1 BLM (1-115), pGEX4T-1 BLM (1-320), pGEX4T-1 BLM (321-530), and pGEX4T-1 BLM (531-660) were obtained by cloning the respective PCR products into the EcoRI/XhoI sites of the vector. pGEX4T-1 BLM (1-1211) and pGEX4T-1 BLM (1-1292) were obtained by cloning the respective PCR products into the XhoI/HindIII sites of the vector. GST Chk2 D347A (kinase dead) and pcDNA3 Flag BLM (S646A) mutants were obtained by site-directed mutagenesis kit (Stratagene).
Kinase and peptide binding assays

Kinase assays with wild-type or kinase-dead Chk1 or Chk2 were carried out as described earlier (8). Chk1 (5 ng) or Chk2 (10 ng) was used at 30°C for 20 minutes. The amounts of BLM and its derivative used in individual experiments, as described in the respective figure legends, were obtained by quantitating the respective Coomassie-visible bands in ImageJ software (NIH). A modified kinase assay used to determine the regions of BLM undergoing phosphorylation in the presence of nuclear extracts had been described earlier (13). For peptide binding assays, the wild-type or the mutant peptide (200 μg) were phosphorylated by the above method. The reactions were stopped with 10% TCA, samples were spotted on P81 phosphocellulose paper (Whatman), extensively washed and incorporated assessed by scintillation counting. UCN-01 (NCI, NIH) was used at 100 nmol/L in both in vitro and cell-based assays. Time of incubation on cells was 2 hours.

Phosphopeptide analysis and phosphoamino analysis

For two-dimensional phosphopeptide maps or phosphorylated amino acid analysis, radiolabeled bands corresponding to the protein(s) of interest were excised from the nitrocellulose membrane and digested with mass spectrometry grade trypsin gold (Promega). The phosphopeptides were analyzed by two-dimensional resolution on thin-layer cellulose plates (19). In cases where the extent of phosphorylation was low, multiple kinase reactions were pooled together so that an equal amount of counts (25,000 cpm) were available for the two-dimensional resolution. Aliquots of the tryptic peptide mixtures were further processed and phosphorylated amino acid analysis was carried out as described (19).

Expression, purification, and interaction of proteins

GST-tagged proteins were expressed according to standard protocols in Escherichia coli at 16°C and subsequently purified by binding to glutathione S-Sepharose (GE Healthcare) for use in interaction studies. Soluble proteins were obtained by eluting the bound proteins with reduced glutathione. pHook Chk1 (wild-type) and pCDZF Chk2 were used for coupled in vitro transcription/translation reactions of Chk1 and Chk2, respectively, using T7 Quick Coupled Transcription/Translation System Kit (Promega). GST-bound target proteins, the expressions of which were visualized by Coomassie and quantitated by ImageJ software (NIH), were incubated with the in vitro–translated interacting partner (one fifth of an entire TNT reaction) for 4 hours at 4°C with constant inversion. Interaction was assayed by autoradiography. BLM was produced in Saccharomyces cerevisiae using the yeast strain JEL1 (donated by Ian Hickson; ref. 20).

Cell culture conditions and treatments

hTERT-immortalized normal human fibroblasts, chromosome 15 minichromosome-corrected BS fibroblasts (referred to as A-15), hTERT immortalized normal human fibroblasts were maintained as described (21). For HU experiments, during immunoprecipitations and immunofluorescence, cells were either left untreated (−HU) or treated (+HU) for 12 hours. The cells were washed and allowed to grow for a further 6 hours (referred to as post-wash, PW). For neocarzinostatin (NCS) treatment, the cells were either left untreated (−NCS) or exposed to the drug for 1 and 6 hours (+NCS). After the 6-hour exposure, the drug was washed off and treatment continued for a further 6 hours (PW). Transfections were carried out with LipofectAMINE 2000 (Invitrogen). Whole cell lysates were made 36 hours posttransfection in radioimmunoprecipitation assay buffer.

Immunoprecipitations, confocal microscopy, and small interfering RNA

Cytoplasmic and nuclear extracts from cells were made using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce). Immunoprecipitations were done as described previously (9) using 1 mg of the nuclear extracts. Immunofluorescence was carried out as described previously (9). For confocal microscopy, the slides were analyzed on a Zeiss 510 Meta system with 63×/1.4 oil immersion objective. The laser lines used were Argon 458/477/488/514 nm (for FITC), DPSS 561 nm (for Texas red), and a Chameleon Ultra autotunable femtosecond laser with a tuning range 690 to 1,050 nm (for 4′,6-diamidino-2-phenylindole). LSM5 software was used for image acquisition. Quantitation was carried out after visualization of at least 200 cells over three experiments. Small interfering RNA (siRNA) transfection for Chk1 (synthesized by Dharmaco) was carried out as previously described (8).

Flow cytometry analysis

Cells at different stages of the cell cycle or after different treatments were subjected to cell cycle analysis in BD FACSCalibur. The data was analyzed either by FloJo or in WinMDI software.

In silico studies

To model peptides in complex with the Chk1 complex structure (11A8), the peptide-bound crystal structure, 2PHK (22), was chosen as a template as it was known to be structurally and functionally similar to Chk1 (23). Chk1 was superimposed onto a 2PHK-MC peptide bound structure using the program ProFit (http://www.bioinf.org.uk/software/profit/) with a rmsd value of 1.372 Å and then MC peptide coordinates were transferred to Chk1, which led to the generation of substrate-bound Chk1. Following standard nomenclature, the site of phosphorylation on the substrate peptide was referred to as P0, whereas the three residues flanking the phosphorylation site on the NH2- and COOH terminus were referred to as P(−3), P(−2), and P(−1) and P(+1), P(+2), and P(+3), respectively. For modeling the 22 known substrates of Chk1, a backbone-dependent rotamer library approach (24) was used for generating side chains corresponding to the known substrates at sites P(−3) to P(+3). The 10 predicted peptides of BLM...
protein were also modeled onto Chk1 kinase using the same approach (24).

The resulting Chk1-peptide complexes were minimized by using CVFF Forcefield and Insight II. Minimizations were carried out using the steepest descent algorithm for initial iterations followed by 5,000 iterations of conjugate gradient algorithm. The convergence criterion was set to rms gradient of 0.001 kcal/mol\AA. The models of Chk1 with 22 known substrates were analyzed for any conserved structural complementarities. Contracting residue pairs between the kinase and the peptide were identified using the criteria of any two atoms of the residue pair being at a distance of ≤4 Å. Another web-based tool—WHAT IF (25), was used to calculate the interactions. The interaction energy (van der Waals attraction/repulsion and electrostatic forces) between the 10 predicted BLM peptides and Chk1 were calculated by using the docking module of Insight II. The contribution of −3 and +1 position to the total binding energy of the peptide was also evaluated. Apart from all-atom interactions, values for 10 BLM peptides were also calculated. The binding energy, based on the total number of contacts between kinase and the peptide, was evaluated using residue-based statistical pair potential (26).

Results

Chk1 and Chk2 phosphorylated BLM on serine and threonine residues in the first 660 amino acids

We had earlier shown that Chk1 phosphorylated BLM in vitro (8). It is also known that Chk1 and Chk2 have overlapping substrate specificities (3). Indeed, like Chk1, wild-type Chk2, but not its kinase-dead counterpart, was able to phosphorylate recombinant wild-type BLM (Fig. 1A). In vitro–translated Chk1 and Chk2 could bind to wild-type BLM and its fragments (Fig. 1B-D). Chk1 preferentially interacted with the NH2-terminal (1-212) region of BLM. However, the central region of BLM, encompassing two fragments (191-660) and (621-1024), also interacted with the kinase (Fig. 1C).

Because BLM is a protein consisting of 1,417 amino acids, we wanted to narrow down the region(s) where the Chk1/Chk2-mediated phosphorylations on BLM possibly occurred. With recombinant BLM fragments and the two kinases, we carried out in vitro phosphorylation on each of the four fragments in the presence of γ\textsuperscript{32}P-ATP. Both BLM (1-212) and BLM (191-660) underwent robust Chk1/Chk2-dependent phosphorylation (Fig. 1E, F). To fine-map the regions of BLM that were highly phosphorylated by Chk1, smaller regions of BLM within the first 660 amino acids were cloned, expressed, and purified (Supplementary Fig. S1A). Although both Chk1 and Chk2 phosphorylated BLM (109-212) and BLM (531-660) to a high extent, Chk2 phosphorylated BLM exclusively between residues (191-320; Supplementary Fig. S1B, C).

Next, we wanted to determine the region(s) of BLM that were phosphorylated by Chk1 in the presence of nuclear extracts prepared from asynchronously growing hTERT-immortalized normal human fibroblasts. Equal amounts of BLM fragments, BLM (1-212), BLM (191-660), BLM (621-1041), and BLM (1001-1417) were subjected to a modified kinase assay in which nuclear extracts were used as the source of kinase. BLM (1-212) and (191-660) were phosphorylated by the kinase present in the nuclear extract of BLM (Supplementary Fig. S1D). The phosphorylation of BLM (1-212) and BLM (191-660) with the nuclear kinase was decreased when Chk1 inhibitor, UCN-01, was included in the reaction mixture (Supplementary Fig. S1E), indicating that the predominant fraction of BLM phosphorylation in the NH2 terminus was dependent on Chk1.

Region within the DExH motif of the helicase negatively regulated Chk1- and Chk2-mediated NH2-terminal phosphorylation on BLM

We hypothesized that an internal stretch of amino acids in BLM may regulate its Chk1/Chk2-mediated NH2-terminal phosphorylation. To test this hypothesis, we cloned, expressed, and purified in E. coli full-length BLM and its seven COOH-terminal fragments (Fig. 2A), and subsequently, carried out phosphorylation with Chk1 or Chk2. BLM (1-660) was highly phosphorylated by both Chk1 and Chk2 (Fig. 2B; Supplementary Fig. S2A), which drastically decreased in BLM (1-800), thereby indicating the presence of a region between amino acids 660 to 800 of the helicase, which negatively regulated Chk1/Chk2-mediated BLM phosphorylation in the first 660 amino acids.

In BLM, the amino acids 683 to 833 encode for DExH motif (27). Our results (Fig. 2B; Supplementary Fig. S2A) indicated that an amino acid sequence within the DExH motif in BLM might negatively regulate the Chk1/Chk2-mediated phosphorylation in the first 660 amino acids of the helicase. To test this hypothesis, we cloned, expressed, and purified GST-BLM (661-800; Fig. 2C, left). GST-BLM (661-800), like GST itself, was not phosphorylated by Chk1. However, the addition of GST-BLM (661-800), but not GST alone, in trans, decreased Chk1/Chk2-mediated BLM (1-660) phosphorylation in a concentration-dependent manner (Fig. 2C, middle and right and Supplementary Fig. S2B). Because BLM (660-800) lies within the ATP-binding/helicase domain, it is possible that the decrease in Chk1/Chk2-mediated phosphorylation was a reflection of sequestration of ATP by this stretch of amino acids. Hence we carried out the kinase reactions in parallel, either without or after preincubation of BLM (661-800) with AMP-PNP, a competitive inhibitor of most ATP-dependent systems. BLM (661-800) could inhibit the Chk1/Chk2-mediated phosphorylation on BLM (1-660), irrespective of AMP-PNP preincubation (Fig. 2D; Supplementary Fig. S2C).

Chk1 phosphorylated BLM at Ser\textsuperscript{646} in vitro

BLM has been implicated as an early responder to replication stress (8, 9). Because Chk1 and its upstream kinase, ATR, are known to be key determinants in the
FIGURE 1. Chk1 and Chk2 phosphorylate BLM in the first 660 amino acids. A, Chk2 phosphorylated wild-type BLM. Recombinant Chk1 [wild-type (WT) or kinase dead (KD)] was incubated with BLM [1-1417; 400 ng (left) and 100 ng, 400 ng (right)] in the presence of γ-32P-ATP. The proteins were resolved by SDS-PAGE and detected by autoradiography. B, left, schematic diagram of full-length BLM and its fragments [1-212, (191-660), (621-1041), and (1001-1417)]. Right, levels of BLM (1-212), BLM (191-660), BLM (621-1041), and BLM (1001-1417) as detected by Coomassie. Approximately 1 μg of protein was loaded in each lane. C, interaction between in vitro–translated S35-radiolabeled Chk1 and equal amounts (5 μg) of the glutathione-Sepharose–bound BLM fragments or GST alone. The amount of bound radioactivity was detected by autoradiography. D, same as C, except that S35-radiolabeled Chk2 was used for interaction with BLM (1-1417). E, Chk1 phosphorylated BLM (1-212) and (1-660). Chk1-dependent kinase assays were carried out with 400 ng of BLM (1-212), BLM (191-660), BLM (621-1041), and BLM (1001-1417). F, same as E, except that Chk2 was used as the kinase to phosphorylate BLM fragments.

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FIGURE 2. Amino acids within the DExH motif negatively regulated the Chk1-mediated NH2-terminal phosphorylation of BLM. A, schematic diagram and relative expression levels of wild-type BLM and its derivatives. Left, full-length BLM (1-1417) and its various fragments (1-660), (1-800), (1-900), (1-1006), (1-1041), (1-1211), (1-1292), and (661-800). The helicase, RQC and HRDC domains are indicated. Right (top), the expression of the BLM (1-660), (1-800), (1-900), (1-1006), (1-1041), (1-1211), (1-1292), and (1-1417) fragments were determined by Coomassie. Approximately 1 μg of protein was loaded in each lane. B, in vitro phosphorylation of full-length BLM and COOH-terminal deletion fragments [i.e., BLM (1-660), (1-800), (1-900), (1-1006), (1-1041), (1-1211) and (1-1292); 400 ng each] in the presence of γ32P-ATP and recombinant Chk1. C, BLM (661-800) negatively regulated the phosphorylation of BLM (1-660). Left, BLM (661-800) was expressed, purified, and checked by Coomassie along with BLM (1-660) and GST. Approximately 1 μg of protein was loaded in each lane. Middle, Chk1-dependent phosphorylation of BLM (1-660; 400 ng) was carried out either alone or in the presence of increasing amounts (50, 100, 200, and 400 ng) of BLM (661-800). As control, phosphorylation was done for GST (400 ng), BLM (661-800; 400 ng), and BLM (1-660; 400 ng) in the presence of GST (400 ng). Right, graph indicates the extent of inhibition of the phosphorylation of BLM (1-660) by GST, Chk1, or Chk2. Points, mean; bars, SD. D, BLM (661-800) inhibited BLM (1-600) phosphorylation even after preincubation with AMP-PNP. Chk1-dependent phosphorylation of BLM (1-660; 400 ng), alone or in presence of BLM (661-800; 400 ng) was carried out either without or after preincubation with BLM (661-800) with AMP-PNP (5 μmol/L).
signal transduction pathway activated in response to stalled replication forks, we decided to determine the sites on BLM which were phosphorylated by Chk1. To have an idea about the number of sites on BLM, which were phosphorylated by Chk1 in vitro, we carried out two-dimensional phosphopeptide map analysis. For this assay, we used recombinant full-length BLM, produced either in E. coli (9) or S. cerevisiae (20). Phosphopeptide map analysis with BLM (1-1417; Fig. 3A) indicated the presence of 25 to 30 phosphopeptides (as verified by color coding; Supplementary Fig. S3A), thereby indicating the presence of at least that many sites at which Chk1 phosphorylated full-length BLM in vitro. Moreover, the similar pattern obtained in the maps derived from recombinant BLM produced in two different hosts indicated a similar three-dimensional structure and folding for both sources of recombinant human BLM. Interestingly, comparison of the phosphopeptide maps of BLM (1-1417), BLM (1-1041), and BLM (1-660) indicated that all phosphopeptides seen in the full-length BLM [except one phosphopeptide present in BLM (1-1417) and BLM (1-1041) as indicated by a red arrow in Fig. 3A] were also present within the first 660 amino acids of the helicase (compare the color codes across peptide maps in Supplementary Fig. S3A), confirming the earlier data (Fig. 1E) that this region of BLM was conserved within smaller BLM fragments when they were phosphorylated by Chk1. Hence, peptide maps were carried out for the smaller fragments of BLM known to be highly phosphorylated by Chk1—i.e., BLM (1-212), BLM (109-212), BLM (191-660), BLM (531-660), and BLM (1-660). Almost all the phosphopeptides seen in BLM (1-212), BLM (109-212), BLM (191-660), and BLM (531-660) were also observed within BLM (1-660) and BLM (1-1417; Fig. 3B; Supplementary Fig. S3B by comparison of color codes). The fragment analysis of Chk1-phosphorylation on BLM thus indicated a possible way by which each phosphorylation site on the helicase could be authenticly mapped on BLM.

Chk1 is known to phosphorylate its substrates on serine and threonine residues, both in vitro and in vivo (3). To check whether Chk1 phosphorylated BLM on serine and/or threonine residues, we carried out phosphoamino acid analysis using purified full-length BLM (1-1417; Fig. 3C). Phosphorylated amino acid analysis indicated that both serine(s), and to a much lesser extent, threonine(s) were phosphorylated on BLM by Chk1.

An analysis of BLM full-length protein sequence indicated the presence of 157 serine and 90 threonine sites. Within the first 660 amino acids, where BLM is supposed to be phosphorylated by Chk1, there were 83 serine and 52 threonine residues. Hence, an in silico analysis was carried out which could potentially help us to understand, in molecular detail, the interactions between Chk1 and short peptide motifs within BLM which may allow a particular site to be preferentially phosphorylated by this kinase. Initially, we used web-based prediction tools such as Kinase-Phos (28), GPS (29), PPSP (30), and Scansite (31) to identify potential sites on BLM which could be phosphorylated by Chk1 (Supplementary Table S1). However, these tools are based purely on motif or amino acid sequences. The number of substrates of Chk1 in the literature was relatively small, and hence, the motif derived from these substrates and used in the sequence-based approaches by the web-based tools may not give robust results. Our recent study (32) found structure-based methods to be superior to sequence-based methods for the identification of substrates for a CHK family of kinases. Therefore, we used a structure-based method to predict the phosphorylation sites in BLM by Chk1. The method extracted information from the three-dimensional structure of the protein–peptide complex and revealed important physicochemical interactions between the kinase and peptide (32). Initially, all the 22 known substrates of Chk1 present in vertebrates (Supplementary Table S2) and reported in PhosphoELM database (33) were modeled in complex with Chk1 using Insight II software. The modeled complexes were then analyzed in detail to identify crucial contact residues, which governed the recognition of the peptide by Chk1. The analysis showed us that two positions, i.e., arginine at P(–3) and hydrophobic residue at P(+1) position in the peptide were more preferred due to its favorable interactions with the Chk1-binding pockets. There were 10 peptides within full-length BLM that matched to the motif [R/K] x x [S/T][hydrophobic] x x (where x indicated any amino acid) which could be potential binders to Chk1. Interestingly, the analysis of the complete BLM sequence by a structure-based kinase substrate prediction tool, MODPROPEP (34), indicated that the 9 out of 10 [R/K] x x [S/T][hydrophobic] x x motif also lied within 30% of the total number of possible Ser/Thr sites on BLM.

Each of these peptides was modeled onto Chk1 and interactions were scored and ranked using residue-based statistical energy potentials by Betancourt and Thirumalai (26). Apart from statistical potentials, binding energy calculations were done for these 10 peptides using all-atom force field. Out of these 10 structural motifs, 6 were within BLM (1-660). Both residue-based statistical pair potentials and all-atom–binding energy values indicated that Ser646 had the highest probability to be a Chk1 substrate (Table 1). Other sites listed in Table 1 could also be potential substrates for Chk1.

We mutated Ser646 in the context of both BLM (1-1041) and BLM (1-660). The mutant proteins were expressed in E. coli, purified, phosphorylated by Chk1, and two-dimensional phosphopeptide map analysis was carried out alongside BLM (1-1417) as control. We found that compared with the BLM (1-1417) peptide map, the intensity of one specific phosphopeptide (indicated by red arrow) was reproducibly decreased in the mutant maps obtained with two different BLM substrates (Fig. 3D). The residual radiolabel in the mutant peptide map was probably due to the low level of Chk1 phosphorylation on another serine residue present within the specific cryptic
FIGURE 3. Chk1 phosphorylated BLM at Ser646 in vitro. A, phosphopeptide maps of human BLM (1-1417; produced either in S. cerevisiae or in E. coli) and BLM fragments (1-1041) and (1-660) phosphorylated in vitro by Chk1. Red arrow, a phosphopeptide present in all except BLM (1-660). Black arrows, the directions in which the phosphopeptides were separated by electrophoresis and chromatography in the first and second dimensions, respectively. B, same as A, except that the following BLM derivatives were used: BLM (1-212), BLM (109-212), BLM (191-660), BLM (531-660), BLM (1-660), and BLM (1-1417). C, phosphoamino acid analysis of BLM (1417). Arrows, directions during the chromatographic runs. Broken circles, the positions of comigrating cold phosphorylated amino acid standards. The position of the origin and products of the partial hydrolysis have been indicated. D, phosphopeptide analysis of BLM (1-1041) and the two mutants BLM (1-1041) S646A and BLM (1-660) S646A. Arrow, phosphopeptide decreased in the mutants. E, peptides (containing either wild-type or mutant Ser646 residue) were phosphorylated with either the wild-type or kinase dead recombinant Chk1. Bound radioactivity was quantitated by scintillation counting.
fragment. Interestingly, the phosphopeptide in which Ser$^{646}$ was present was also observed in the phosphopeptide map analysis of BLM (191-660) and BLM (531-660; Fig. 3B; Supplementary Fig. S3B), thereby validating the robustness of our assay system. Together, these results indicated that Chk1 phosphorylated BLM at Ser$^{646}$ in vitro.

To verify whether Chk1 could phosphorylate BLM at Ser$^{646}$, we examined the context of a specific peptide, wild-type and mutant peptides spanning BLM (641-651), containing a mismatch at Ser$^{646}$ were generated. Kinase assays were carried out with Chk1, either wild-type or mutant, and the extent of phosphorylation on the two peptides was determined using the peptide-binding assay (Fig. 3E). Wild-type Chk1 phosphorylated the wild-type peptide but not the mutant one, whereas mutant Chk1 phosphorylated the peptides to a basal level. The above results indicated that in vitro Chk1 phosphorylation on BLMSer$^{646}$ was a specific phenomenon.

BLM Ser$^{646}$ phosphorylation was lost after DNA damage

Next, we wanted to determine whether Ser$^{646}$ phosphorylation on BLM was observed in vivo. Immortalized cells obtained from BS patients were used along with chromosome 15–complemented BS cells (A-15). The cells were either left untreated or treated with Chk1 inhibitor UCN-01 for 2 hours, which did not lead to any drastic change in the cell cycle profile (Fig. 4A, left) or change in the expression level of endogenous BLM in the nucleus (Fig. 4A, middle). We generated a new phosphospecific polyclonal antibody against a BLM peptide phosphorylated at Ser$^{646}$. Because the levels of pSer$^{646}$BLM after direct Western analysis were not equivocally apparent, we enriched the phosphorylated moiety by immunoprecipitating endogenous BLM from nuclear extracts of A-15 cells grown in the absence or presence of UCN-01. Phosphorylation on BLM at Ser$^{646}$ was decreased when A-15 was treated for 2 hours with UCN-01 (Fig. 4A, right), indicating that Ser$^{646}$ was phosphorylated in a Chk1-dependent manner on asynchronously growing cells. A similar decrease of phosphorylation on BLM at Ser$^{646}$ was observed when asynchronously growing A-15 cells were depleted of Chk1 by Chk1 siRNA transfection (Supplementary Fig. S4A).

The presence of BLM phosphorylation at Ser$^{646}$ in asynchronous culture led us to investigate its status after stalled replication forks. A-15 and BS cells were grown either asynchronously (−HU) or in the presence of hydroxyurea (+HU) for 12 hours. The cells were also kept for 6 hours post-removal of HU (referred to as post-wash, PW), which allowed the cells to proceed to G2-M phase (Supplementary Fig. S4B). The levels of BLM increased after stalling of the replication forks and in G2-M phase (Supplementary Fig. S4C, top). It has been reported that replication arrest lead to the generation of double-strand breaks (35). It was found that BLM was phosphorylated at Ser$^{646}$ only under asynchronous conditions and this phosphorylation was lost when the cells were treated with HU (Fig. 4B, bottom). Loss of Ser$^{646}$ phosphorylation after treatment with celfin intestinal alkaline phosphatase acted as a specificity control for the Ser$^{646}$BLM phosphorylation under asynchronous conditions.

Because double-strand breaks are the end result of stalled replication (35), we hypothesized that loss of Ser$^{646}$ phosphorylation on BLM should also be observed after the exposure of cells to double-strand break–inducers like neocarzinostatin (NCS). Hence, we treated A-15 cells with

Table 1. Ranking of BLM peptides potentially phosphorylated by Chk1 as obtained by in silico analysis

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Ser/Thr position</th>
<th>Peptide sequence</th>
<th>Residue-based statistical pair potential scores (BT)</th>
<th>Binding energy calculated using Insight II (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>VDW</td>
<td>ELE</td>
</tr>
<tr>
<td>1</td>
<td>Ser$^{646}$</td>
<td>RFQSLSF</td>
<td>–7.72</td>
<td>–66.274</td>
</tr>
<tr>
<td>3</td>
<td>Thr$^{581}$</td>
<td>KSSTAID</td>
<td>–5.03</td>
<td>–55.567</td>
</tr>
<tr>
<td>4</td>
<td>Ser$^{1361}$</td>
<td>KGGSATC</td>
<td>–4.81</td>
<td>–43.757</td>
</tr>
<tr>
<td>5</td>
<td>Ser$^{375}$</td>
<td>KSSSIG</td>
<td>–3.79</td>
<td>–53.314</td>
</tr>
<tr>
<td>7</td>
<td>Thr$^{350}$</td>
<td>RRKTASS</td>
<td>–3.01</td>
<td>–55.531</td>
</tr>
<tr>
<td>8</td>
<td>Ser$^{367}$</td>
<td>RQISLQQ</td>
<td>–2.72</td>
<td>–66.311</td>
</tr>
<tr>
<td>9</td>
<td>Ser$^{502}$</td>
<td>RLSSAATK</td>
<td>–2.4</td>
<td>–52.816</td>
</tr>
<tr>
<td>10</td>
<td>Thr$^{182}$</td>
<td>RVSTAOK</td>
<td>–2.08</td>
<td>–53.744</td>
</tr>
</tbody>
</table>

NOTE: The residue-based statistical pair potential scores and all-atom binding energies for the peptides are listed. The peptides are ranked based on their residue-based statistical pair potential scores. VDW represents the contribution due to van der Waals interaction, whereas ELE represents the contribution due to electrostatic interaction.
FIGURE 4. BLM phosphorylation at Ser646 decreased after DNA damage. A, left, cell cycle profile of A-15 and BS, both grown asynchronously, and A-15 treated with UCN-01 for 2 h. Middle, nuclear extracts (50 μg) from A-15, grown in the above two conditions, and BS (grown asynchronously) were subjected to SDS-PAGE and Western blotting to determine the level of BLM and hsp90. Right, nuclear extracts (1 mg) from A-15 and BS were immunoprecipitated with BLM antibody and the immunoprecipitates subjected to Western blotting with either BLM or pSer646BLM antibody. BLM was immunoprecipitated from nuclear extracts (1 mg) obtained from BS and A-15 cells (−HU, +HU, PW) with anti-BLM antibody. The immunoprecipitates were probed with either BLM (top) or pSer646BLM antibody (bottom). Immunoprecipitates from A-15 cells were additionally incubated with calf intestinal alkaline phosphatase (10 units) for 30 min before Western blotting to check for the specificity of pSer646BLM signal under −HU condition. *, a cross-reactive band. C, immunoprecipitation with anti-BLM antibody was carried out on nuclear extracts isolated from A-15 cells obtained from different conditions. The immunoprecipitates were probed for the presence of BLM, pSer646BLM, nucleolin, PML isoforms, and Nbs1. D, immunoprecipitation with anti-pSer646BLM antibody was carried out on nuclear extracts isolated from A-15 cells obtained from different conditions. The immunoprecipitates were probed for the presence of pSer646BLM, BLM, and nucleolin.
NCS for 1 or 6 hours, and also allowed a subsequent recovery for 6 hours after wash-off. Such a treatment regimen did not lead to much alteration in the levels of endogenous BLM, but led to the accumulation of γ-H2AX (Supplementary Fig. S4D). Immunoprecipitation of endogenous BLM from the nuclear extracts of A-15 cells revealed that Ser646 phosphorylation was much reduced within 1 hour after NCS treatment and was no longer detectable after 6 hours (Fig. 4C).

Because Ser646 phosphorylation on BLM was present only in the asynchronous cultures, we wanted to investigate whether the loss of this specific phosphorylation coordinated with the cellular relocalization of BLM. For this purpose, A-15 cells were either left untreated or treated with HU for 12 hours and coimmunostained with BLM and pSer646BLM antibody. Under asynchronous conditions, both BLM and pSer646BLM colocalized (Fig. 5A, a) within the PML NBs and nucleolus (Fig. 5A, b and c). After HU treatment, BLM localization was decreased in the PML NBs and nucleolus and increased at the sites of stalled replication where the helicase colocalized with proteins involved in sensing and resolution of DNA damage such as RAD51, 53BP1 (8, 21), and Nbs1 (Fig. 5A, c; data not shown). Very little staining of pSer646BLM was observed in cells treated with HU (Fig. 5A, d). Similar loss of pSer646BLM staining within the PML NBs and nucleolus was also observed after treatment of A-15 cells with NCS (data not shown). Incidently, robust Ser646 phosphorylation on BLM was observed within 6 hours after NCS wash-off (Fig. 4C), which coincided with the colocalization of BLM within the PML NBs and nucleolus (data not shown). The lack of pSer646BLM signal in the PW condition after HU treatment (Fig. 4B, bottom), points to the possibility of differences in the dynamics of BLM relocalization post-NCS and –HU treatment. The above results indicated that the relocalization of BLM post-DNA damage correlated with the loss of Ser646 phosphorylation. The relocalization of BLM was verified by its immunoprecipitation from A-15 cells. Although the binding of BLM to both nucleolin and PML isoforms decreased after DNA damage, its in vivo association with Nbs1 increased within 1 hour and persisted up to 6 hours after DNA damage (Fig. 4C). To further determine whether Ser646 phosphorylation was one of the prerequisites for the relocalization of BLM from the nucleolus after DNA damage, we carried out communoprecipitation with pSer646BLM antibodies. Loss of BLM Ser646 phosphorylation increased after DNA damage and correlated with decreased binding to nucleolin (Fig. 4D).

To further validate the above results, mutational analysis was carried out with overexpressed Flag-tagged wild-type or BLM (S646A) variants in Cos cells (Supplementary Fig. S4E). UCN-01 treatment for 2 hours did not cause any change in the expression levels of the transfected BLM, PML isoforms, or nucleolin. Immunoprecipitation was carried out with either nucleolin (Fig. 5B) or PML (Fig. 5C). Wild-type BLM but not the S646A mutant interacted with both nucleolin and PML isoforms in asynchronous conditions (Fig. 5B and C). However, both nucleolin-BLM and PML-BLM interactions were much decreased after UCN-01 treatment, indicating that Ser646 constitutive phosphorylation might be one of the post-translational events that regulated BLM localization under asynchronous conditions.

Discussion

In this communication, we have provided evidence that apart from Chk1, Chk2 also phosphorylated BLM in vitro in the NH2-terminal 660 amino acids (Fig. 1; Supplementary Fig. S1). Chk1/Chk2-mediated phosphorylation of BLM was regulated by an internal stretch of amino acids present within the DExH motif of the helicase (Fig. 2; Supplementary Fig. S2). Using biochemical, in silico, and cell biology techniques, we have shown that Chk1 could phosphorylate BLM at Ser646 in a constitutive manner in vivo (Figs. 4 and 5). Phosphorylation on BLM at Ser646 was only present in asynchronous cultures and decreased rapidly after DNA damage. Loss of this phosphorylation event led to a decrease of BLM binding to nucleolin and PML isoforms, diminished accumulation in the nucleolus and PML NBs, coinciding with its (i.e., BLM’s) simultaneous relocalization to the sites of the DNA damage, and binding with DNA sensor protein, Nbs1 (Figs. 4C, D, and 5). Thus, the results indicated that Ser646 phosphorylation could act as a marker to determine the activity status of the helicase. Constitutive phosphorylation of H2AX, ATM, and Chk2 has been found in human cancerous tissues and shown to be associated with precancerous lesions (36, 37). Hence, it will be of interest to know the status of BLMSer646 phosphorylation in different stages and grades of tissues obtained from cancer patients.

Events involving phosphorylation cascades are spatially and temporarily regulated. SMC3 (structural maintenance of chromosomes subunit 3) controls the activity of chromosomes during cell division and also plays important roles in stabilizing cells’ genetic information and repairing damaged DNA. SMC3 is phosphorylated at Ser1067 and Ser1083 in vivo. Phosphorylation at Ser1083 was IR induced, dependent on ATM and NBS1, and was required for intra-S phase checkpoint. ATM-dependent phosphorylation at Ser1083 was in turn dependent on the constitutive phosphorylation of Ser1067 by CK2 (38). Similarly, it is possible that phosphorylation at Ser646 may either coordinate or even regulate other posttranslational events on BLM, which may happen after DNA damage. These post-translational events might involve other Chk1-dependent phosphorylation events on BLM. Indeed, around 25 to 30 phosphopeptides were present in the peptide map of Chk1-phosphorylated wild-type BLM (Fig. 3A; Supplementary Fig. S3A), indicating that Chk1 possibly phosphorylated BLM on multiple residues. The Chk1-mediated phosphorylation events on BLM may mutually regulate each other temporally and thereby modulate BLM functions during signal transduction cascades.
DExH motif–containing proteins are more common among the RNA helicases. Mutations in the conserved residues of DExH motif revealed its role in ATPase and RNA helicase activities (39). DExH motif is also present in the DNA helicases of the SF2 superfamily, to which BLM belongs (27). DExH motif in BLM, which extended from amino acids 683 to 833, was contained within the ATP-binding region in BLM. During this study, we have provided

**FIGURE 5.** Ser^646 phosphorylation of BLM regulates its localization in PML NBs and nucleolin. A, A-15 cells were either left untreated (−HU) or treated with HU (+HU) for 12 h. Immunofluorescence was carried out with antibodies against (a, d) BLM/pSer^646BLM, (b) pSer^646BLM/PML, (c) pSer^646BLM/nucleolin, and (e) BLM/Nbs1. The nucleus was stained by 4,6-diamidino-2-phenylindole (DAPI). Combined indicates the merged image from the red and green channels. The numbers indicate the percentage of cells having similar colocalization. B and C, immunoprecipitations were carried out with either antinucleolin (B) or anti-PML (C) antibody on cell extracts (1 mg) obtained after transfecting Cos cells with either pcDNA3 Flag BLM (wild-type) or pcDNA3 Flag BLM (S646A). The immunoprecipitates were probed for the presence of nucleolin and Flag, i.e., BLM (for B) or PML isoforms and Flag, i.e., BLM (for C).
evidence that amino acids (661-800) within the DExH motif negatively regulated the Chk1/Chk2-mediated NH2-terminal phosphorylation of BLM (Fig. 2; Supplementary Fig. S2). The addition of BLM (661-800) in trans inhibited Chk1/Chk2 phosphorylation (Fig. 2D; Supplementary Fig. S2C), thereby indicating that in the native conformation, the DExH motif and the NH2-terminal region of the protein were probably in close proximity, which allowed the former to regulate the phosphorylation event in the latter. Hence, a regulatory circuitry probably exist in vitro which may coordinate and temporaroly control vital biochemical processes mediated by the helicase domain of BLM and the phosphorylation events mediated by Chk1/Chk2. We have recently shown that BLM could enhance the ATPase function of RAD54 and thereby stimulate RAD54-mediated chromatin remodeling (9). Because DExH RNA helicases have been shown to remodel ribonucleoprotein complexes (40), it will be tempting to speculate that Chk1/Chk2 phosphorylation in general, and Ser646 phosphorylation on BLM may regulate the effect of the helicase during disruption of RAD51 nucleofilaments. Future research will determine how Chk1/Chk2-mediated phosphorylations in the NH2 terminus of BLM indeed affected the functions of the helicase during homologous recombination.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

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