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Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent Phosphorylation of Chk1 on Ser-317 in Response to Ionizing Radiation*

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In mammals, the ATM (ataxia-telangiectasia-mutated) and ATR (ATM and Rad3-related) protein kinases function as critical regulators of the cellular DNA damage response. The checkpoint functions of ATR and ATM are mediated, in part, by a pair of checkpoint effector kinases termed Chk1 and Chk2. In mammalian cells, evidence has been presented that Chk1 is devoted to the ATR signaling pathway and is modified by ATR in response to replication inhibition and UV-induced damage, whereas Chk2 functions primarily through ATM in response to ionizing radiation (IR), suggesting that Chk2 and Chk1 might have evolved to channel the DNA damage signal from ATM and ATR, respectively. We demonstrate here that the ATR-Chk1 and ATM-Chk2 pathways are not parallel branches of the DNA damage response pathway but instead show a high degree of cross-talk and connectivity. ATM does in fact signal to Chk1 in response to IR. Phosphorylation of Chk1 on Ser-317 in response to IR is ATM-dependent. We also show that functional NBS1 is required for phosphorylation of Chk1, indicating that NBS1 might facilitate the access of Chk1 to ATM at the sites of DNA damage. Abrogation of Chk1 expression by RNA interference resulted in defects in IR-induced S and G_2/M phase checkpoints; however, the overexpression of phosphorylation site mutant (S317A, S345A or S317A/S345A double mutant) Chk1 failed to interfere with these checkpoints. Surprisingly, the kinase-dead Chk1 (D130A) also failed to abrogate the S and G_2 checkpoint through any obvious dominant negative effect toward endogenous Chk1. Therefore, further studies will be required to assess the contribution made by phosphorylation events to Chk1 regulation. Overall, the data presented in the study challenge the model in which Chk1 only functions downstream from ATR and indicate that ATM does signal to Chk1. In addition, this study also demonstrates that Chk1 is essential for IR-induced inhibition of DNA synthesis and the G_2/M checkpoint.

The detection of DNA damage in mammalian cells induces an array of responses that result in cell cycle arrest, DNA repair, gene transcription, and, in some instances, cell death (1). In mammals, the ataxia-telangiectasia-mutated (ATM)[1] and ATM and Rad3-related (ATR) protein kinases function as critical regulators of the cellular DNA damage response. ATM and ATR are Ser/Thr-Gln-directed protein kinases with overlapping substrate specificities that are activated in response to distinct as well as partially overlapping types of genotoxic stimuli (2, 3). Despite their structural similarity and overlapping substrate specificities, ATR and ATM are functionally non-redundant protein kinases. Cells from A-T patients or *ATM*-nullizygous mice are exquisitely sensitive to ionizing radiation (IR) and other agents that induce double strand breaks and are defective in activating IR-induced G_1/S, S phase, and G_2/M checkpoints (4, 5), whereas checkpoint responses to UV light and base-damaging agents are normal in A-T cells. In contrast to $ATM^{-/-}$ mice, which are viable, ATR-deficient mice die early during embryogenesis, and the conditional knockout of *ATR* gene function in human cells leads to a loss of cellular viability (6–8). ATR is required for the G_2/M DNA damage checkpoint as well as the DNA replication checkpoint, which suppresses mitosis in the presence of unreplicated DNA (9). ATR mediates responses to a broad spectrum of genotoxic stimuli, including IR, DNA replication inhibitors (*e.g.* hydroxyurea or HU), UV light, and agents such as *cis*-platinum that induce DNA inter-strand crosslinks (9, 10). A common denominator of these agents is the ability to induce transient or prolonged DNA replication fork stalling during S phase. This stalled replication fork may be a critical trigger for ATR signaling potential.

The checkpoint functions of ATR and ATM are mediated, in part, by a pair of checkpoint effector kinases termed Chk1 and Chk2/Cds1 (1, 2, 3). Although structurally distinct, Chk1 and Chk2 are functionally related kinases that phosphorylate an overlapping pool of cellular substrates (11). In mammalian cells, evidence has been presented that Chk2 and Chk1 have apparently evolved to channel DNA damage signals from ATM and ATR, respectively. This is based on reports that Chk1 appears to be devoted to the ATR signaling pathway and is modified by ATR in response to replication inhibition and UV-induced damage, whereas Chk2 functions primarily through

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[1] The abbreviations used are: ATM, ataxia-telangiectasia-mutated protein kinase; ATR, ATM and Rad3-related protein kinase; IR, ionizing radiation; HU, hydroxyurea; A-T, ataxia-telangiectasia; NBS, Nijmegan breakage syndrome; siRNA, small interfering RNA; Gy, gray.

ATM in response to IR (12–14). Here, we have investigated the contribution of ATM in the regulation of Chk1 after IR. We demonstrate that Chk1 is a downstream target of ATM and is phosphorylated on Ser-317 in response to IR. Reduction of Chk1 expression by RNA interference results in a defect in regulation of the S and G_2 phase checkpoints; however cells overexpressing the phosphorylation site mutants Chk1 were not compromised in their ability to arrest in S and G_2 following IR.

MATERIALS AND METHODS

Cell Lines, Lysate Preparation and Immunoblotting—Control, A-T, and NBS lymphoblastoid cell lines were grown in RPMI 1640 with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were exposed to genotoxic agents (gamma, UV radiation, or HU) and, unless otherwise stated, harvested at the indicated time points. Cell extracts were prepared by lysis in universal immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM sodium fluoride, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 0.2% Triton X-100, and 0.3% Nonidet-P40). Twenty-five micrograms of whole cell extract was loaded per lane on 7.5% SDS-PAGE gels. Samples were transferred to nitrocellulose using carbonate buffer at 30 V for 3 h, and the membranes were probed with appropriate antibodies.

Rabbit polyclonal anti-phosphopeptide antisera against ATM/ATR phosphorylation sites in Chk1 (Ser-317 and Ser-345) were raised by immunizing rabbits with the keyhole limpet hemocyanin-conjugated phosphopeptides NVKYSS-PhosphoSer-QPEPRT (for Ser-317) and VQGISF-PhosphoSer-QPTCPD (for Ser-345). Bleeds were assayed by enzyme-linked immunosorbent assay, and the serum was collected on days corresponding to the peak antibody response and affinity purified against the same peptide. Anti-Chk1 monoclonal and anti-cyclin B antibodies were from Santa Cruz Biotechnology, and anti-S15p53 was from New England Biolabs.

Plasmid Constructs and siRNA Oligos—ATM-complemented cells (15) and NBS1-complemented (16) cells have been described previously. Kinase-dead Chk1 (D130A, D148E), phosphorylation site Chk1 mutants (S317A, S345A, S317A/S345A, S317D, S345D, S317D/S345D) were constructed using the Quick Change mutagenesis kit (Stratagene) by using FLAG-tagged Chk1 in PCI-Neo vector (Promega) as a template. The details of the primers are available from the authors on request.

RNA interference of Chk1 was performed using 5′-UCGUGAGC-GUUUGUUGAAC-3′. siRNA specific to HSP70 served as a negative control (GCACAUCAGCCAGAACAAG). The oligos were purchased from Dharmacon Research, Boulder, CO. A siRNA duplex was annealed using the protocol supplied by the company, and transfection was carried out using the OligofectAMINE reagent (Invitrogen) according to the manufacturer's recommendations. Cell lysates were prepared 48 h after transfection.

Synchronization of Cells—Cells were synchronized by double thymidine block (17). Briefly, 2.5 mM thymidine was added for 16 h, cells were washed twice with phosphate-buffered saline and released in fresh media containing 24 μM thymidine and 24 μM deoxycytidine for 10 h. Cells were reblocked in 2.5 mM thymidine for 16 h. At this point, the cells were washed twice with phosphate-buffered saline and placed into normal medium. This point was designated *t* 0, the point at which 80% of cells are in G_1/S by propidium iodide-flow cytometry analysis, and cells were harvested at 4 h (mid S phase), 7 h (G_2 phase), and 9.5 h (G_2 + M phase). At each time point the cells were either left untreated or treated with 6 Gy of IR for 30 min. Total cell extracts were prepared as described above and blotted with the respective antibodies.

G_2/M Checkpoint—Cells were mock transfected or transfected with various Chk1 constructs or Chk1 siRNAs. Forty-eight hours later, cells were mock irradiated or exposed to 3 Gy of IR. Cells were harvested 1 h after IR and fixed in 70% ethanol at −20 °C. After centrifugation, the cell pellet was suspended in 100 μl of phosphate-buffered saline containing 0.25 μg of phospho-histone H3 antibody and 1% bovine serum albumin followed by staining with a fluorescein isothiocyanate-labeled secondary antibody as described previously (18). After 1 h of incubation at room temperature in the dark, the cells were stained with phosphatidylinositol, and cellular fluorescence was measured by BD Biosciences FACScan. The data was analyzed using Cell Quest software.

Intra-S Phase Checkpoint—Suppression of DNA synthesis after DNA damage was assessed as described (19). Cells were labeled with 10 nCi of [^{14}C]thymidine for 24 h. The isotope was then washed away during the transfection procedure, and the cells were transiently transfected with siRNA oligos Chk1 or co-transfected with pCMV CD-20 and various Chk1 constructs at the ratio of 1:10 and incubated without [^{14}C]thymidine for 24 h. Cells were then irradiated with 10 Gy, and, 45 min post-irradiation, [^{3}H]thymidine (2.5 μCi/ml) was added. Fifteen minutes after the addition of [^{3}H]thymidine, cells were harvested and fixed in 70% methanol either directly or after isolation with anti-CD-20-coupled Dynabeads as described (19), and radioactivity was measured in an liquid scintillation counter. The DNA synthesis was estimated by the ratios of ^{3}H to ^{14}C and expressed as a percentage of control values.

RESULTS

Chk1 Ser-317 Phosphorylation Is Defective in ATM- and NBS-deficient Cells—To delineate the relationship between ATM and Chk1, we studied Chk1 mobility shift in control and AT cells treated with IR. In control lymphoblastoid cells (C3ABR and C2ABR), a small fraction of the Chk1 protein from IR-treated cells showed a reproducible reduction in mobility compared with untreated cells, whereas most of the AT cell lines (L3, AT1ABR, AT3ABR) tested failed to show that shift in mobility (Fig 1*A*). Chk1 modification post UV irradiation was quite comparable in AT and control lymphoblastoid cell lines (Fig. 1*A*). Previous studies have shown that this modification of Chk1 after DNA damage is because of phosphorylation. Therefore, we examined the *in vivo* phosphorylation status of Chk1 in AT and control cells with or without prior exposure to irradiation. AT and control cells were metabolically labeled with ^{32}P for 2 h immediately after exposure to 6 Gy of irradiation. Chk1 was immunoprecipitated from the cell lysates and fractionated by 10% SDS-PAGE, and radiolabeled Chk1 was detected by autoradiography. Chk1 in non-irradiated control and A-T cells appears to be already phosphorylated, and this phosphorylation was greatly enhanced (3-fold) 2 h after IR in control cells. In contrast, there was a barely detectable increase in ^{32}P-labeled Chk1 from IR-treated AT cells (Fig. 1*B*). Earlier studies have mapped two damage-induced sites (Ser-317 and Ser-345) of phosphorylation in Chk1, and ATR has been shown to phosphorylate Chk1 on Ser-345 and Ser-317 primarily in response to replication inhibition and UV (12, 14). Therefore, to examine Chk1 phosphorylation we generated rabbit polyclonal antibodies against a synthetic polypeptide consisting of phosphorylated Ser-317 or Ser-345. We were unable to detect Ser-345 phosphorylation of Chk1 in response to IR in lymphoblastoid or fibroblast cell lines from control individuals. However, significant phosphorylation of Ser-317 in Chk1 was observed in response to various damaging agents in control cells. A-T cells were defective in rapid phosphorylation of Chk1 on Ser-317 in response to IR (6 or 10 Gy), but at a high dose of IR (20 Gy) or after HU and UV, Chk1 phosphorylation in AT and control cells was quite comparable (Fig 1*C*, *top panel*). The extent of phosphorylation of Chk1 was quite comparable with the phosphorylation of p53, a widely accepted target of ATM (Fig 1*C*, *bottom panel*). The time course analysis revealed that Chk1 phosphorylation was induced in control cells within 30 min post-IR (6 Gy), whereas in ATM-deficient cells it became evident by 2 h and was still markedly reduced compared with controls at 6 h after IR (Fig. 1*D*). Defective IR-induced Chk1 phosphorylation in A-T cells was corrected by the introduction of ATM cDNA; therefore, it is indeed ATM-dependent (Fig. 1*E*). Ser-317 phosphorylation of Chk1 was also defective (Fig. 1*F*) in lymphoblastoid cells derived from individuals either homozygous or heterozygous for ATM mutation (T7271G), which is known to confer a high risk of breast cancer (20, 21). We have shown previously that this mutant form of ATM is quite stable but intrinsically defective as a kinase (21). The mutant allele has been shown to act as a dominant negative manner so that the wild-type ATM is unable to function in the presence of

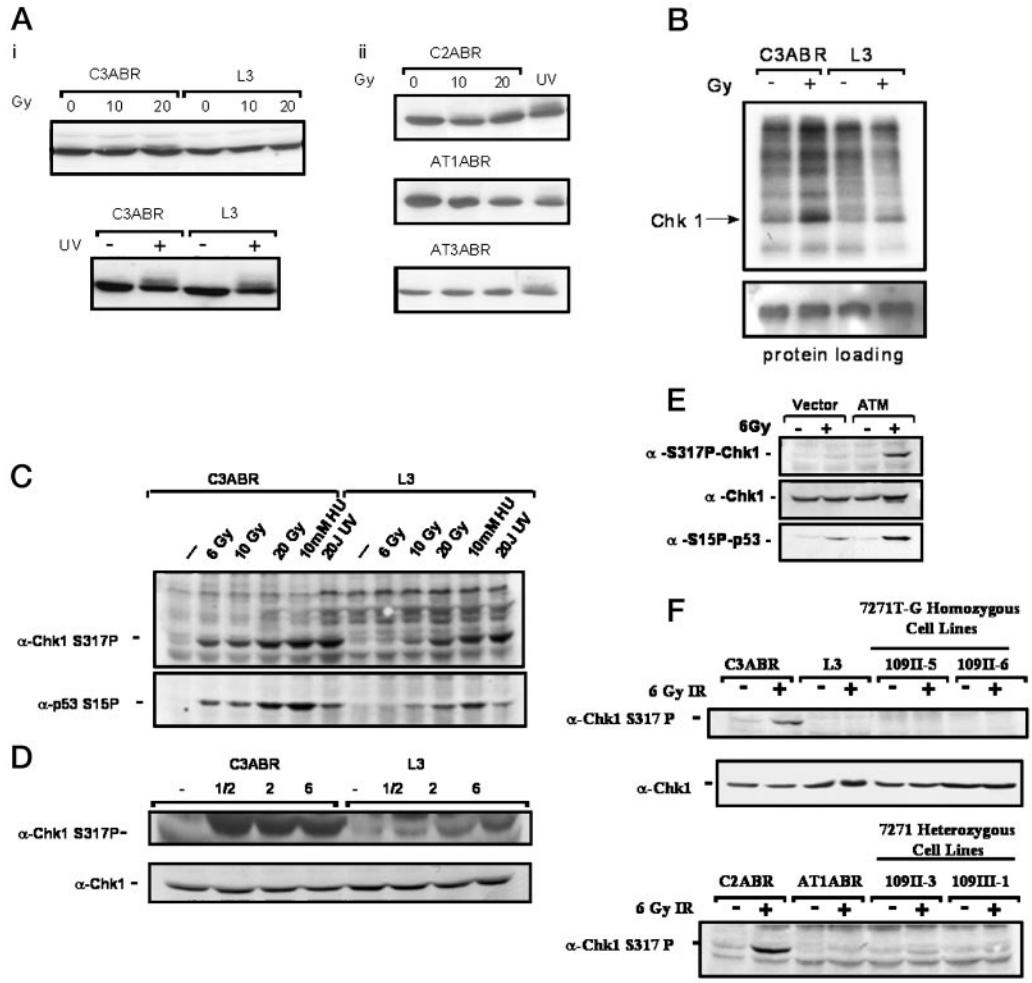

FIG. 1. **ATM dependence of Chk1 phosphorylation in response to IR.** *A*, Chk1 mobility shift in control and A-T cells in response to IR. Asynchronously growing cultures were exposed to IR at indicated dosage or UV (50J/m^2), and cell lysates were immunoblotted with anti-Chk1 antibody. *C3ABR*, control lymphoblastoid cell lines; *AT1ABR* and *AT3ABR*, AT cell lines. *L3* is a lymphoblastoid cell line established from a North African Jewish A-T patient (obtained from Y. Shiloh). *B*, *in vivo* phosphorylation of Chk1 in response to IR. Mock-treated or IR-treated cells were incubated with ^{32}P-labeled inorganic phosphate. Anti-Chk1 immunoprecipitates were autoradiographed. *C*, analysis of Chk1 phosphorylation on Ser-317 after various DNA damaging agents. Total cell lysates were prepared 1 h after treatments as indicated above the lanes and immunoblotted with an antibody specific for phospho Ser-317 Chk1 (*α-Chk1 S317P*; *upper panel*) or phospho Ser-15 p53 (*α-p53 S15P*; *bottom panel*). *D*, time course of phosphorylation of Chk1 on Ser-317 in response to IR. Cells were irradiated at 6 Gy, and total cell lysates, prepared at the indicated time points, were immunoblotted with an antibody specific for phospho Ser-317 Chk1 (*α-Chk1 S317P*; *upper panel*) or total Chk1 (*α-Chk1*; *lower panel*). *E*, Ser-317 phosphorylation in an ATM-reconstituted AT cell line. ATM-null (AT221JE-T) cells containing either vector alone or vector expressing ATM were irradiated at 6 Gy and harvested 1 h later. Total cell extracts were probed with anti-Ser-317-Chk1 (*α-S317P-Chk1*; *top panel*), anti-total-Chk1 (*α-Chk1*; *middle panel*), or anti-Ser-15-p53 antibody (*α-S15P-p53*; *bottom panel*). *F*, Ser-317 phosphorylation in lymphoblastoid cell lines from individuals, either homozygous (*109II-5* and *109II-6*) or heterozygous (*109II-3* and *109III-1*) for the ATM T7271G mutation. Cells were exposed to ionizing radiation and harvested 1 h later. Cell extracts were probed with anti-phospho-Ser-317 antibody (*α-Chk1 S317 P*) or anti-total Chk1 antibody (*α-Chk1*).

mutant protein. The defective Ser-317 phosphorylation in a heterozygous carrier of T7271G mutation is related to this dominant negative effect (Fig. 1*F*).

Evidence from yeast and vertebrate systems has shown that the activation of Chk1 and Chk2 requires a large number of additional gene products that participate in the detection, initiation, and propagation of checkpoint signals (22). Additionally, radiation-induced phosphorylation of certain ATM substrates such as SMC1 has been reported to be defective in NBS cells (23, 24), whereas phosphorylation of others (p53, BRCA1, and Rad9) has been reported to occur normally in NBS cell lines (16, 25). Therefore, we examined the dependence of Chk1 phosphorylation on NBS1. Dramatically reduced phosphorylation of Chk1 in response to IR was also observed in cells deficient in NBS1 as compared with control cells (Fig. 2*A*), but after HU and UV, Chk1 phosphorylation in NBS1-deficient cells and control cells was comparable (Fig. 2*B*). Phosphorylation of Chk1 was restored with the introduction of NBS1 cDNA in NBS-deficient fibroblasts (Fig 2*C*). NBS1 is a target of ATM, and ATM phosphorylates NBS1 on Ser-343. This phosphorylation is required for activation of the S phase checkpoint. We tested the possible requirement for the phosphorylated form of NBS1 in Chk1 phosphorylation by using the NBS cells that were complemented with wild-type NBS1 and the ATM phosphorylation site mutant (S343A) NBS1 (Fig. 2*C*, *top panel*). Interestingly, Chk1 expression was up-regulated (5-fold) by overexpression of S343A mutant NBS1 (Fig. 2*C*, *bottom panel*); however, the dependence of Ser-317 phosphorylation on NBS1 phosphorylation was not clear (Fig. 2*C*, *middle panel*). Quantification of data indicated that phosphorylation, relative to the amount of Chk1 protein, was reduced in S343A-overexpressing cells.

To test whether activation of Chk1 after IR required a functional ATM protein, we assayed Chk1 activation in ATM-null

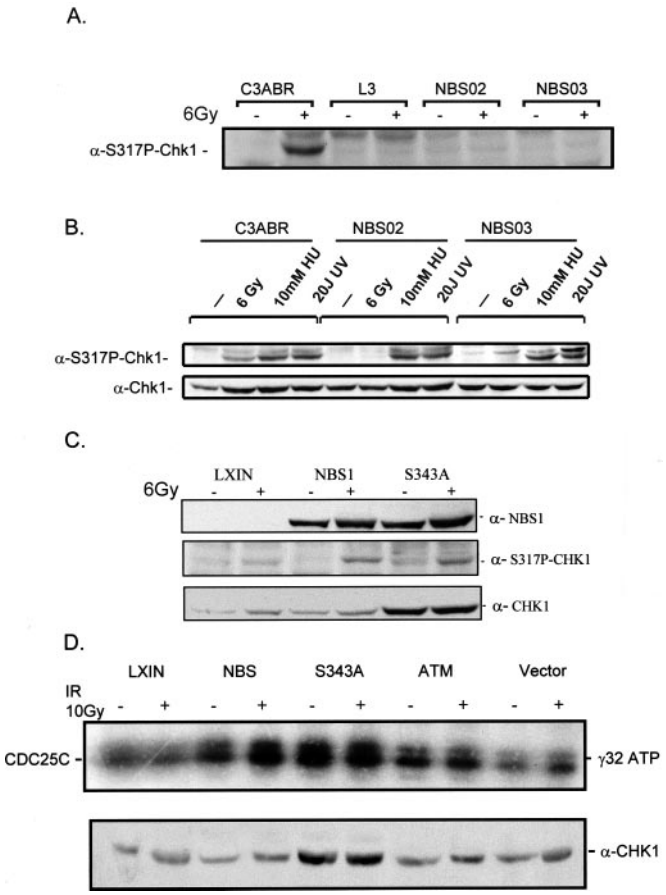

FIG. 2. **NBS1 dependence of Chk1 phosphorylation on Ser-317 in response to IR.** *A*, control (*C3ABR*), ATM-deficient (*L3*) and NBS-deficient cell lines (*NBS02* and *NBS03*) were exposed to 6 Gy of IR and harvested 1 h later. Cell extracts were probed with anti-phospho Ser-317 (*α-S317P-Chk1*). *B*, UV- and HU-induced phosphorylation of Chk1 on Ser-317 does not require functional NBS1. Cells were treated with various DNA-damaging agents as indicated above the lane and analyzed by immunoblotting with anti-phospho Ser-317. *C*, Ser-317 phosphorylation in an NBS1-reconstituted NBS-deficient cell line (ILB1). Cells were transfected with retroviral vector alone (*LXIN*), full-length NBS1, or a S343A mutant NBS1 and immunoblotted with anti-NBS1 (*top panel*), anti-phospho Ser-317 (*middle panel*), or anti-total Chk1 (*bottom panel*). *D*, Chk1 activation in ATM- and NBS1-reconstituted cells. Cells were untreated or treated with radiation of 6 Gy, and kinase assays were performed on immunoprecipitated Chk1 using GST-Cdc25 (amino acids 200–256) as a substrate (*top panel*), and levels of Chk1 were visualized by Western blotting with anti-Chk1 antibody (*bottom panel*).

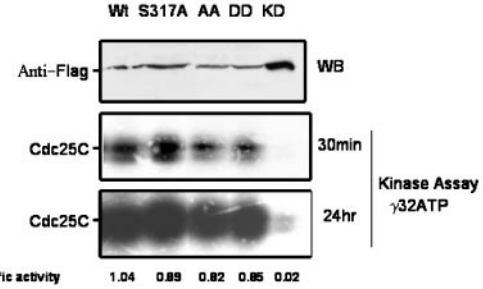

FIG. 3. **Protein kinase activity of Chk1 phosphorylation site mutants.** 293T cells were transfected with FLAG-tagged Chk1 wild-type construct (*Wt*), S317A mutant, S317A/S345A mutant (*AA*), S317D/S345D mutant (*DD*), and D130A mutant (*KD*, kinase dead). Lysates were prepared and immunoprecipitated with anti-FLAG antibody. Precipitates were tested for their ability to phosphorylate GST-Cdc25C residues 200–256 (*middle* and *bottom panels* showing autoradiographs after 30 min and 24 h of exposure), and levels of Chk1 wild-type and various mutants were determined by immunoblotting with anti-FLAG antibody (*top panel*). Specific activity of each construct was determined by quantitating GST-Cdc25C phosphorylation by densitometry and normalizing it to the amount of Chk1 analyzed by immunoblotting with anti-FLAG.

cells complemented with empty vector or vector encoding wild-type ATM. Treating ATM-null cells with radiation had no effect on Chk1 activity and, interestingly, cells expressing ATM had slightly higher basal levels of Chk1 activity compared with ATM-null cells. IR treatment in the presence of ATM had a small effect on the activation of Chk1 (Fig. 2*D*). Similarly, we studied Chk1 activation after IR in NBS1 defective cells with or without complementation with NBS1 and failed to observe the NBS1 dependence of Chk1 activation after IR; however, like the ATM reconstituted cells, NBS1 reconstituted cells showed higher basal activity compared with retroviral vector only (LXIN) transfected cells.

Chk1 and G_2/M Checkpoint—To address the functional significance of the phosphorylation of Chk1, we mutated Ser-317 and Ser-345 singly or doubly to either alanine or aspartate. The rationale for generating these mutations was that the alanine mutants, which cannot be phosphorylated, might not be active if Chk1 phosphorylation is important for its activity, and the negatively charged carboxyl group of aspartate may potentially mimic phosphoserine and would therefore be expected to exhibit similar functions to phosphorylated Chk1. Human embryonic kidney 293T cells were transfected with FLAG-tagged wild-type Chk1, phosphorylation site mutants, and kinase-dead Chk1 to initially determine whether intrinsic kinase activity of Chk1 was altered as a consequence of amino acid substitution. Thirty-six hours after transfection, cells were either left unirradiated or irradiated with 6 Gy of IR. Lysates were prepared and immunoprecipitated with anti-FLAG antibody and analyzed for Chk1 protein and Chk1 kinase activity. As seen in Fig. 3, each phosphorylation site mutant of Chk1 (both alanine and aspartate) had specific activity quite comparable with wild-type Chk1, whereas kinase dead-Chk1 (D130A) had negligible activity in this assay. Furthermore, the activity of the constructs examined above remained unchanged after treatment with IR (data not shown). These results indicate that the phosphorylation site mutants of Chk1 do not affect the intrinsic activity of Chk1 to a great extent.

Earlier studies using small molecule inhibitors of Chk1 (SB-218078 and UCN-01) have demonstrated a role for Chk1 in the regulation of the G_2/M checkpoint (26, 27). However, it is difficult to attribute their effects to Chk1 inhibition, as these inhibitors do inhibit other kinases as well. We therefore chose to address the requirement for Chk1 in G_2 checkpoint signaling more specifically using an siRNA knockout of Chk1 expression. HeLa cells were incubated with Chk1-specific siRNA, and the Chk1 expression was significantly reduced in treated cells (Fig. 4*C*). Both control and siRNA treated HeLa cells were examined for their ability to delay in G_2 at 1 h after exposure to 6 Gy of IR by flow cytometric assessment of anti-phospho-histone H3 antibody to distinguish mitotic cells from G_2 cells. Control cells treated with nonspecific siRNA showed a 43–48% decrease in the number of cells entering mitosis (Fig. 4, *A* and *B*). The Chk1 siRNA-treated samples showed no detectable reduction in mitotic index following 1 h post IR (Fig. 4, *A* and *B*). These results suggest that Chk1 is required for the delay in mitotic entry after IR. To determine whether phosphorylation events contributed to G_2/M checkpoint regulation by Chk1, we tested whether an IR-induced G_2 checkpoint could be abrogated by overexpression of alanine phosphorylation site mutants of Chk1. Wild-type Chk1 and phosphorylation site mutant transfected cells (Ser-317, Ser-345, and double mutations involving Ser-317 and Ser-345) showed between 80 and 70% reduction in

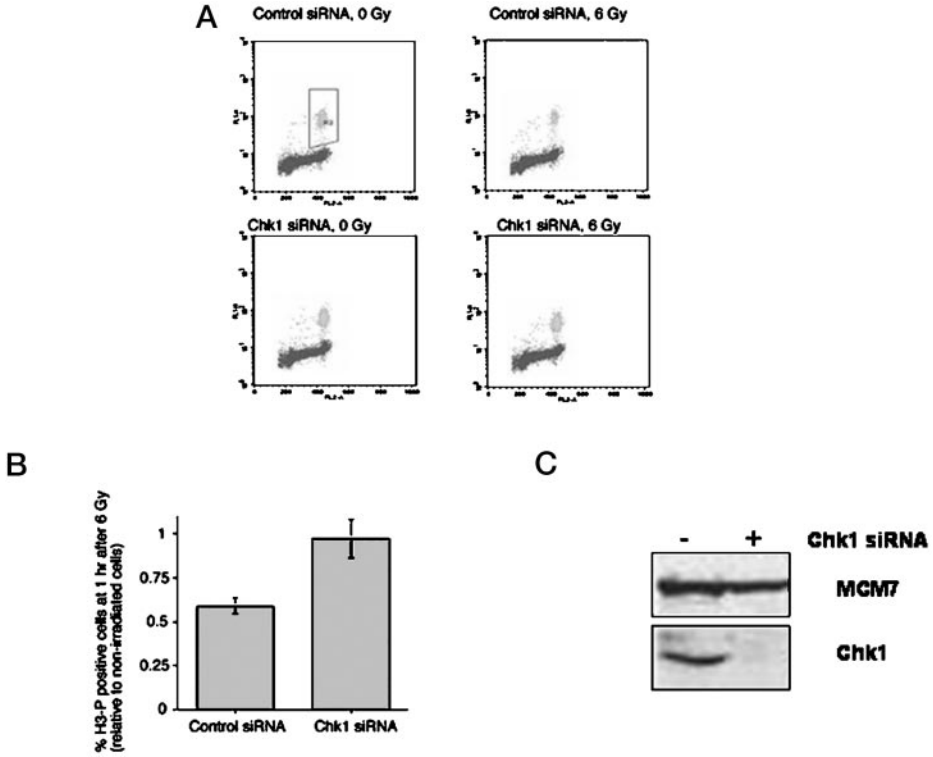

FIG. 4. **Chk1 regulates IR-induced G_2/M checkpoint.** HeLa cells were treated with siRNA Chk1 oligos for 48 h. *A*, cells were either untreated or irradiated with 6 Gy, then incubated for 1 h, stained for mitotic index (as described under "Materials and Methods"), and analyzed by flow cytometry. *B*, quantitation of results of three independent experiments described in *A*. *C*, Chk1 expression in cells treated with siRNA Chk1 oligos. Note that MCM7 expression was used as control for equal protein loading.

the number of mitotic cells. Cells expressing the kinase inactive Chk1 (D130A and D148E) were also normal in the ability to delay in G_2 following IR. Under the same experimental conditions, expression of kinase-dead ATM showed only a 20% reduction in the suppression of mitotic entry after IR. Taken together, these results show that, although Chk1 is required for G_2 arrest, the precise role, if any, of the phosphorylations at Ser-317 and Ser-345 in G_2 arrest remains to be established.

Chk1 and S Phase Checkpoint—To test whether phosphorylation of Chk1 on Ser-317 occurred during normal cell cycle progression, we synchronized HeLa cells at the G_1/S boundary by double thymidine block and followed their progression through the cell cycle after release into the fresh medium. Chk1 was phosphorylated on Ser-317 at the G_1/S boundary and mid S phase of normal S phase progression; however, no phosphorylation was evident in cells in G_2 or G_2/M (Fig. 5*A*). In contrast, damage-induced phosphorylation of Chk1 on Ser-317 was observed at all cell cycle stages. Similar results were obtained with MCF-7 cells synchronized by serum starvation and released into the fresh medium (data not shown). To determine whether S phase phosphorylation of Chk1 is also required for an IR-induced S phase checkpoint, we used U20S cells, which have been shown previously to be proficient in both branches of the S phase checkpoint (ATM/NBS1 and ATM/Chk2/Cdc25A branch). Vector-transfected U2OS cells irradiated at 10 Gy showed ~50% inhibition of their DNA synthesis within 1 h post IR, as monitored by [^{3}H]thymidine incorporation into DNA. Cells expressing kinase dead Chk1 (D130A) or phospho mutant Chk1 (S317A, S345A, and S317A/S345A) showed comparable levels of suppression of DNA synthesis after IR (Fig. 5*B*). Thus, these experiments did not show any obvious negative effect of these mutants on the checkpoint function in the presence of endogenous Chk1. On the other hand, down-modulation of endogenous Chk1 by siRNA treatment, followed by IR and monitoring of the S phase checkpoint, revealed a radiation-resistant DNA synthesis phenotype (Fig. 5*B*), a hallmark of checkpoint failure. Identical results showing the radio-resistant DNA synthesis phenotype were obtained when the activity

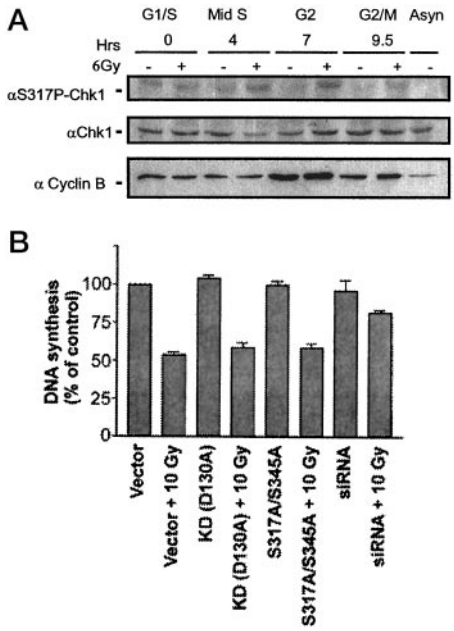

FIG. 5. **Chk1 regulates IR-induced suppression of DNA synthesis, but phosphorylation site mutants are dispensable.** *A*, cell cycle-dependent phosphorylation of Chk1 on Ser-317 during the unperturbed cell cycle and in response to DNA damage. Cell were synchronized by a double thymidine block and collected at various time points during the cell cycle as indicated under "Materials and Methods" and blotted for immunostaining with respective antibodies. *B*, cells were transfected with vector alone, Chk1 phosphorylation site mutants (S317A/S345A), or kinase-dead (*KD*) Chk1 construct or treated with siRNA Chk1 oligos. Replicative DNA synthesis was assessed 1 h after exposure to 10 Gy. *Error bars* represent the standard deviation of duplicate samples.

of Chk1 was inhibited by the chemical inhibitor UCN01 (data not shown). Thus, analogous to the requirement of Chk1 for the activation of the G_2/M checkpoint, Chk1 is also essential for the IR-induced inhibition of DNA synthesis.

DISCUSSION

The protein kinase Chk1 is phosphorylated at Ser-317 and Ser-345 in response to DNA damage. There has been accumulating evidence that these phosphorylation events are regulated by ATR (12–14). ATR shares overlapping substrate specificities with ATM *in vitro*, but *in vivo* it is regulated in response to different damaging agents. Recent studies have indicated that, in mammalian cells, ATR and ATM might represent two parallel branches of the DNA damage response pathway and that Chk1 and Chk2 in mammals might have evolved to signal DNA damage from ATR and ATM, respectively (12, 13). The ATM/Chk2 pathway is activated, principally, by double-stranded DNA breaks, whereas the ATR/Chk1 pathway primarily responds to lesions caused by UV and DNA replication block. Here we provide evidence for cross-talk between the two pathways by demonstrating that ATM does target Chk1 in response to IR. We demonstrate that phosphorylation of Chk1 on Ser-317 in response to IR is severely compromised in ATM-deficient cells and that ectopic expression of ATM corrects this defect. However, Chk1 phosphorylation in response to HU and UV is ATM-independent, possibly catalyzed by ATR, as indicated by earlier studies that have shown that expression of a kinase-inactive form of ATR interfered with UV-induced phosphorylation of Chk1 (13, 14).

Our results establish that the *NBS1* gene product is required for optimal IR-induced phosphorylation of Chk1 on Ser-317 but is dispensable for its phosphorylation in response to HU and UV. The simplest interpretation of the data is that NBS1 assists ATM in targeting some of its substrates. This explanation is consistent with ATM and NBS1 dependence of SMC1 phosphorylation in response to IR (23, 24). Normal BRCA1, p53, and H2AX phosphorylation (16, 28) in NBS1-deficient cells, previously reported by us and others, suggests that NBS1-independent mechanisms might promote the phosphorylation of these substrates by ATM. Notably, p53 and BRCA1 can both be efficiently phosphorylated by ATM *in vitro*, whereas Chk1 is a poor ATM substrate *in vitro* (data not shown).

There has been controversy in the literature as to whether Chk1 activity is increased after DNA damage; however, after repeated attempts we have not been able to demonstrate any consistent increase in activity after IR. Furthermore, we do not see a defect in Chk1 kinase activation in ATM-deficient or NBS-deficient cells in response to IR. To understand whether phosphorylation of Chk1 is required for its checkpoint function, we overexpressed phosphorylation site mutants of Chk1 either singly (S317A and S345A) or in combination to study their effects on G_2/M and S phase checkpoint. Both phosphorylation site mutants were active as a protein kinase with or without prior exposure to IR. Our results demonstrate that the phosphorylation site mutants do not interfere with the checkpoint control, yet the existence of some additional site(s) of phosphorylation in Chk1, potentially capable of mediating the checkpoint function, is not very likely given that these two sites of phosphorylation are the only two Chk1 sites conserved across other species, including fission yeast, *Caenorhabditis elegans*, *Xenopus*, and *Drosophila* (13). Furthermore, similar to the phospho mutants, we were unable to demonstrate a dominant negative effect through overexpression of kinase-inactive Chk1 (D130A or D148E). These findings raise the possibility that Chk1 activation does not depend on its dimerization and that the interactions with its substrates are very dynamic, with a rapid turnover of interaction. Consequently, the kinase-inactive form of the protein may not be capable of sequestering targets into non-functional complexes. Thus, it might not be possible to demonstrate a dominant negative effect of Chk1 phospho mutants in overexpression studies. Therefore, the significance of the phosphorylation sites can only be addressed by comparing the abilities of wild-type and phospho mutant Chk1 to restore the checkpoint functions in Chk1-deficient cells. The major problem with these experiments is the lack of availability of Chk1-null cells, as Chk1 is an essential gene in mice (14, 29). Nevertheless, we have shown that the reduction of Chk1 expression in mammalian cells by siRNA does lead to a defect in S and G_2/M checkpoint control, which is consistent with a recently published report (30). These Chk1-deficient cells might be suitable for complementation studies with wild-type or Chk1 mutant constructs. The challenge here will be to modify various Chk1 constructs to make them resistant to inhibition by siRNA, which will be the goal of future experiments.

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Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent Phosphorylation of Chk1 on Ser-317 in Response to Ionizing Radiation

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