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

Received for publication, October 23, 2002, and in revised form, February 13, 2003
Published, JBC Papers in Press, February 14, 2003, DOI 10.1074/jbc.M210862200

Magtouf Gatei, Katie Sloper, Claus Sorensen, Randi Syljuasen, Jacob Falck, Karen Hobson, Kienan Savage, Jiri Lukas, Bin-Bing Zhou, Jiri Bartek, and Kum Kum Khanna

From the Queensland Institute of Medical Research, Post Office Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia, the Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100, Copenhagen, Denmark, and Incyte Genomics, Newark, Delaware 19714

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 ATM 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 G2/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 G2 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 G2/M checkpoint.

*This work was supported by funds from the National Health and Medical Research Council, the Queensland Cancer Fund, the Sylvia & Charles Viertel Foundation, Alfred Benzon's Fund, and the Danish Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1To whom correspondence should be addressed. Tel.: 61-7-33626338; Fax: 61-7-33620106; E-mail: kumkumK@qimr.edu.au.

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) 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 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 G2/S, S phase, and G2/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 G2/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

1The 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, Nijmegen 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 G2 phase checkpoints; however cells overexpressing the phosphorylation site mutants Chk1 were not compromised in their ability to arrest in S and G2 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-phosphoepitope 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-QPPERT (for Ser-317) and VQGFQP-phosphoSer-QPMPDD (for Ser-345). Beads 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 against the same peptide. Anti-Chk1 monoclonal and anti-cyclin B antibodies were from Santa Cruz Biotechnology, and anti-S15p53 was against the same peptide.

**Fluorometric Constructs and siRNA Oligos—**ATM-complemented constructs (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-GUUUGUGAAG-3'. siRNA specific to H2P70 served as a negative control (GCACAUCGCGAGAAG). The oligos were purchased from Dynal. Cells were transfected using the OligofectAMINE reagent (Invitrogen) according to the protocol supplied by the company, and transfection was carried out using the Quick Change mutagenesis kit (Stratagene) 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 is greatly enhanced (at least 2 fold) 2 h after irradiation. In contrast, there was a barely detectable increase in 32P-labeled Chk1 from IR-treated A-T cells (Fig. 1B). Early 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 1C, top panel). The extent of phosphorylation of Chk1 was quite comparable with the phosphorylation of p53, a widely accepted target of ATM (Fig 1C, 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 1D). Defective IR-induced Chk1 phosphorylation in A-T cells was corrected by the introduction of ATM cDNA; therefore, it is indeed ATM-dependent (Fig 1E).

Ser-317 phosphorylation of Chk1 was also defective (Fig 1F) 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...
The defective Ser-317 phosphorylation in a heterozygous carrier of T7271G mutation is related to this dominant negative effect (Fig. 1F).

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. 2A), but after HU and UV, Chk1 phosphorylation in NBS1-deficient cells and control cells was comparable (Fig. 2B). Phosphorylation of Chk1 was restored with the introduction of NBS1 cDNA in NBS-deficient fibroblasts (Fig. 2C).

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

**Phosphorylation of Chk1 on Ser-317 in Response to IR**

**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 (50 J/m²), 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 32P-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-3 and 109II-6) or heterozygous (109II-3 and 109II-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 S317P) or anti-total Chk1 antibody (α-Chk1).
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. 2D). 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 G2/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 (D190A) 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 G2/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 G2 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. 4C). Both control and siRNA treated HeLa cells were examined for their ability to delay in G2 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 G2 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 G2/M checkpoint regulation by Chk1, we tested whether an IR-induced G2 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. 2. NBS1 dependence of Chk1 phosphorylation on Ser-317 in response to IR.](image)

![Fig. 3. Protein kinase activity of Chk1 phosphorylation site mutants.](image)
the number of mitotic cells. Cells expressing the kinase inactive Chk1 (D130A and D148E) were also normal in the ability to delay in G2 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 G2 arrest, the precise role, if any, of the phosphorylations at Ser-317 and Ser-345 in G2 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 G1/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 G1/S boundary and mid S phase of normal S phase progression; however, no phosphorylation was evident in cells in G2 or G2/M (Fig. 5A). 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 U2OS 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 [3H]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. 5B). 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. 5B), a hallmark of checkpoint failure. Identical results showing the radio-resistant DNA synthesis phenotype were obtained when the activity of Chk1 was inhibited by the chemical inhibitor UCN01 (data not shown). Thus, analogous to the requirement of Chk1 for the activation of the G2/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 are the only two Chk1 sites conserved across species. Phosphorylation site mutants do not interfere with the checkpoint activity of 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.

Acknowledgments—We thank Malcolm Taylor for supplying cell lines from the T7271G families, Yosef Shiloh for providing the A-T cell line (L3) and ATM complemented cells, Patrick Concannon for supplying the NBS1 complemented cells, and Meri Hoekstra for the wild-type Chk1 expression construct.

REFERENCES

Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent Phosphorylation of Chk1 on Ser-317 in Response to Ionizing Radiation
Magtouf Gatei, Katie Sloper, Claus Sörensen, Randi Syljuåsen, Jacob Falck, Karen Hobson, Kienan Savage, Jiří Lukas, Bin-Bing Zhou, Jiří Bartek and Kum Kum Khanna

doi: 10.1074/jbc.M210862200 originally published online February 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210862200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 20 of which can be accessed free at
http://www.jbc.org/content/278/17/14806.full.html#ref-list-1