Regulation of CHK2 by DNA-dependent Protein Kinase*

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Chk2 is a critical mediator of diverse cellular responses to DNA damage. Activation of Chk2 by DNA damage requires phosphorylation at sites including Thr68. In earlier work, we found that an activity present in rabbit reticulocyte lysates phosphorylates and activates Chk2. We now find that hypophosphorylated Chk2 can be phosphorylated at Thr68 by various subcellular fractions of HEK293 cells. This activity is sensitive to the phosphatidylinositol 3'-kinase-like kinase inhibitor wortmannin, but not to caffeine. DNA enhances the Chk2 phosphorylation by cellular fractions in vitro. The wortmannin-sensitive Chk2 kinase activity is present in fractions from ATM-deficient cells. In contrast, Chk2 was not efficiently phosphorylated at Thr68 in vitro by fractions from cells with a defective DNA-dependent protein kinase (DNA-PK) catalytic subunit. Chk2 is phosphorylated by purified DNA-PK in vitro. Endogenous Chk2 coimmunoprecipitates Ku70 and Ku80. In a series of matched cell lines having and lacking functional DNA-PK, Chk2 activation by exposure of cells to ionizing radiation, or to camptothecin was consistently diminished in the absence of DNA-PK. Down-regulation of DNA-PKcs by either siRNA or a chemical inhibitor attenuated radiation-induced Chk2 phosphorylation. Ionizing radiation-induced Chk2 phosphorylation was wortmannin-sensitive in ATM-defective cells with depleted ATR. These results suggest that DNA-PK augments ATM and ATR in activation of Chk2 by DNA damage.

DNA damage checkpoint pathways sense genomic lesions and induce cell cycle arrest, transcriptional induction of repair-related genes, and/or apoptosis. Two members of the phosphatidylinositol 3'-kinase-like kinase (PIKK) family, ATM and ATR, play central roles in DNA damage checkpoint signal transduction. ATM is activated mainly by DNA double-strand breaks (DSBs). Cells from patients with ataxia telangiectasia (AT), a disease caused by ATM mutations, are hypersensitive to ionizing radiation (IR), and have defects in IR-induced G1, S, and G2/M cell cycle arrest. ATR responds to a wider range of signals, including ultraviolet light (UV)-induced damage, DSBs, and stalled replication forks. ATM and ATR each phosphorylate several substrates. These include Chk1 and Chk2, which are important effector kinases with overlapping functions.

Under basal conditions, ATM exists as an inactive form in dimers or oligomers. Irradiation induces the rapid intermolecular autophosphorylation of ATM, which leads to dissociation of ATM dimers and the activation of ATM kinase activity (2). The principal kinase relaying signals initiated by ATM appears to be Chk2. UV or replication blockade causes the phosphorylation of Chk2 independent of ATM, possibly through ATR (3, 4). Activated Chk2 mediates IR-induced inhibition of DNA synthesis through the phosphorylation of Cdc25A, which triggers the ubiquitination and proteasomal degradation of Cdc25A (5). In addition, Chk2 contributes to G2/M arrest through inhibitory phosphorylation of the mitosis-promoting Cdc25C phosphatase (6). By phosphorylating p53, Chk2 also helps maintain sustained G1, G2/M arrest, and apoptosis (7).

Additional substrates of Chk2 include the tumor suppressor Brca1, PML (promyelocytic leukemia gene product), and the transcription factor E2F1 (8–10).

Chk2 function requires several evolutionarily conserved domains. They include an N-terminal SCD (SQ/TQ cluster domain), which contains multiple consensus SQ/TQ phosphorylation sites for PIKKs, a FHA (forkhead-associated domain) domain, which binds to phosphopeptides, and a C-terminal kinase domain (7). ATM phosphorylates Chk2 at Thr68 (3, 4), which is followed by oligomerization of Chk2 through FHA domain/phospho-SCD interactions, autophosphorylation and activation (11–13). Phosphorylation of Thr68, located in the SCD, and the integrity of the FHA domain are required for full activation of Chk2, probably because they promote oligomerization (3, 4, 11, 13). This seems to be a multistep process, in which phosphorylation of the SCD by PIKKs or other Chk2 molecules permits cross-phosphorylation and activation of Chk2 at sites within the activation loop of the kinase domain (Thr382 and Thr387).

A third PIKK family member DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase composed of a large catalytic subunit, DNA-PKcs of ~460 kDa, and a regulatory component, the Ku70-Ku80 heterodimer (14). DNA-PK is activated by association with DNA. Ku binds to DNA ends directly, and then recruits DNA-PKcs (14). The kinase activity of DNA-PK is required for NHEJ (nonhomologous end joining) (15), the predominant mechanism for DSB repair in mammalian cells. NHEJ is also crucial for V(D)J recombination in development of the immune system. Cells and animals defective in DNA-PK are deficient in DSB repair and V(D)J recombination, and, thus, are hypersensitive to IR and are immunodeficient (14).

The protein kinase activity of DNA-PK is up-regulated by DNA damage induced by IR, UV, and V(D)J recombination (16). However, the role of DNA-PK in DNA damage checkpoint signaling is controversial. Phosphorylation of replication protein A (RPA) by DNA-PK may be involved in DNA damage-induced replication arrest (17). DNA-PK selectively regulates...
p53-mediated apoptosis, but not cell cycle arrest, after exposure to IR (18–20). c-Abl is activated in an ATM-dependent manner by exposure to IR (21, 22). DNA-PK can phosphorylate and activate c-Abl, while c-Abl regulates DNA-PK in a negative feedback loop by phosphorylating DNA-PK and diminishing its binding to DNA (23). Hence, the down-regulation of DNA-PK by c-Abl is dependent on ATM (24).

Many proteins have been identified that are phosphorylated by DNA-PK in vitro, including XRCC4, p53 and Sp1, but few have been corroborated by in vivo analysis (14). We have found that DNA-PK is the major constituent of an activity present in extracts of mammalian cells that phosphorylates Chk2. Our results suggest that hypophosphorylated Chk2 can be phosphorylated at Thr68 by DNA-PK in vitro. Likewise, DNA-PK appears to be involved in activation of Chk2 in response to DNA damage in vivo. We also found that Chk2 can form protein complexes with Ku70 and Ku80. These results support the model that the PIKKs ATM, ATR, and DNA-PK collaboratively transduce DNA damage signals to downstream kinases including Chk2.

MATERIALS AND METHODS

Cell Culture and Fractionation—V3-H15 (DNA-PK-defective Chinese hamster ovary (CHO) cell line V3 with reconstituted wild-type DNA-PKc), and V3-KA4 (V3 with reconstituted kinase-defective DNA-PKc) were generous gifts from D. Chen (15). Immortalized A-T fibroblasts A-T22JE-T stably transfected with pEBSt encoding full-length ATM were obtained from NIGMS Human Genetic Mutant Cell Repository. Other cell lines were from American Type Culture Collection. M059K and M059J cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium (1:1 mixture) supplemented with 2.5 mM L-glutamine and 10% fetal bovine serum. V3-H15 and V3-KA4 were maintained in α-MEM supplemented with 10% fetal bovine serum, 1 mM sodium orthovanadate, and 0.5 mM dithiothreitol. The washed chromatin-enriched fraction (P) (Fig. 1A, lanes 1–4) was separated from the cytoplasmic fraction (S1) by centrifugation (5 min, 3,300 × g). Nuclei were washed with solution A (hypotonic buffer plus 0.34M sucrose and 10% glycerol), resuspended in solution A, and 107 cells were lysed by Dounce homogenization. The chromatin fraction was greatly diminished by preincubation first for 1 h at 30 °C with either kinase buffer A only or cell fractions in kinase buffer A, in the presence of an ATM-regenerating system (5 mM ATP, 70 mM creatine phosphate, 0.1 mg/ml creatine kinase). Alternatively, in vitro translation products were incubated first for 1 h at 30 °C with either kinase buffer A only or cell fractions in kinase buffer A, in the presence of an ATM-regenerating system. Then NETN buffer and anti-FLAG M2 affinity beads were added and immunoprecipitations performed. Purified DNA-PK was obtained from Promega.

Immunoprecipitation—U2-OS cells were lysed with lysis buffer (NETN buffer supplemented with 20 mM NaF, 1 mM sodium orthovanadate and protease inhibitor mixture). For immunoprecipitation, in vitro translated FLAG-Chk2 was mixed with U2-OS lysates, and rotated at 4 °C for 1 h. Then, anti-FLAG M2 affinity beads were added and immunoprecipitations performed for 3–4 h.

In vitro coimmunoprecipitation of Chk2 and Ku70/Ku80, MCF-7 cells were treated with 10 Gy of IR or were mock-irradiated. After 30 min of recovery, cells were lysed with lysis buffer. Endogenous Chk2 was immunoprecipitated with a mixture of two rabbit polyclonal anti-Chk2 antibodies.

Experimental Treatments—Cells were irradiated in a Mark I 137Cs irradiator (Shepard) or treated with 1 µM camptothecin (Sigma). Where indicated, cells were pretreated with NU7026 (2-(morpholin-4-yl)-benzimidazole) (4–10 µM) for 1 h (Promega). The luciferase GL2 siRNA (5′-CGTACGGGAAATTCGCTTTTCAAT-3′) was used as control.

In Vitro Kinase Assays—Chk2 was immunoprecipitated with rabbit polyclonal antibodies from either V3-H15 or V3-KA4 cell lysates made with lysis buffer. Immunoprecipitated Chk2 was incubated at 30 °C for 10 min in 1× kinase buffer B (20 mM HEPES pH 7.5, 70 mM KCl, 10 mM MgCl2, 5 mM MnCl2, 1 mM dithiothreitol) with 2 µCi nonradioactive ATP and 10 µCi of [γ-32P]ATP (7,500 Ci/mmol, Amer sham Biosciences). Figures present results from a minimum of two trials.

RESULTS

An Activity That Phosphorylates Chk2 in Vitro Is Sensitive to Wortmannin and Is Enhanced by DNA—Thr68/Ser286 and Thr68 are prefered PIKK phosphorylation sites located within the SCD in the N terminus of Chk2 (7). ATM phosphorylation of Chk2 Thr68 is required for full activation of Chk2 (3, 4). Chk2 produced in vitro using a wheat germ-derived translation system is hypophosphorylated (as judged by electrophoretic mobility), is not recognized by phosphospecific antibodies for phospho-Thr297/Ser296 or phospho-Thr68, and has minimal auto-phosphorylation activity (11). Incubation of hypophosphorylated wheat germ-translated Chk2 with rabbit reticulocyte lysates enhances Thr68 phosphorylation and kinase activity (11). To further characterize cellular activities that phosphorylate Chk2, we prepared subcellular fractions from HEK293 cells. Incubation of wheat germ-translated hypophosphorylated Chk2 with cytoplasmic (S1), soluble nuclear (S2), or chromatin (P) fractions from HEK293 cells in the presence of an ATM-regenerating system resulted in Thr68 phosphorylation (Fig. 1A, lanes 1–4). The most efficient phosphorylation of Chk2 occurred after incubation with the chromatin-enriched fraction (P) (Fig. 1A, lane 4). Thr68 phosphorylation of Chk2 by the chromatin fraction was greatly diminished by preincubation of chromatin with wortmannin (Fig. 1A, lanes 6 and 7), a specific PIKK inhibitor with stronger inhibitory effects on DNA-PK and ATM than on ATR at the concentrations used (32). In contrast, preincubating chromatin fractions with another PIKK inhibitor, caffeine, at concentrations from 1 mM to 10 mM did not inhibit the Thr68 phosphorylation of Chk2 (Fig.
To further explore the roles of different PIKKs in Chk2 phosphorylation, hypophosphorylated Chk2 was incubated with the chromatin-enriched fraction from AT cells that were either transfected with vector only, or were reconstituted with wild-type ATM. Reintroduction of ATM into AT cells restored the normal sensitivity to ionizing radiation and intra-S phase checkpoint (25). Chromatin fractions from ATM-defective cells phosphorylated Chk2 at Thr68 to a similar extent as cells with reconstituted ATM (Fig. 1B, compare lane 1 to lane 4). This indicated that ATM is not the major PIKK phosphorylating Chk2 in this assay. Moreover, wortmannin inhibited the phosphorylation of Chk2 not only by chromatin preparations from cells with wild-type ATM (Fig. 1B, lanes 5 and 6), but also by fractions from cells with defective ATM (Fig. 1B, lanes 2 and 3), suggesting that another target of wortmannin is responsible for Chk2 phosphorylation.

DNA-PKcs can bind to DNA in vitro in the absence of Ku, but its protein kinase activity is stimulated severalfold by the interaction with DNA-bound Ku (14). Addition of DNA enhanced Thr68 phosphorylation of both wild-type and kinase-defective Chk2 by the subcellular fractions, especially cytosol (S1) and chromatin-enriched (P) fractions (Fig. 1C). Because DNA-PK is the major DNA-activated PIKK (14, 16), these data further support a role of DNA-PK in the phosphorylation of Chk2. The phosphorylation of Chk2 in vitro by ATM purified from human placenta was not enhanced by the addition of DNA (34). The effect of DNA on ATR activity is controversial. Some groups did not observe a stimulatory effect of DNA on the kinase activity of ATR (35), whereas other groups have reported that DNA activates ATR (36, 37).

Cellular Fractions from DNA-PK-defective Cells Do Not Phosphorylate Chk2 Efficiently in Vitro—Because these results suggested that DNA-PK is the wortmannin-sensitive kinase for Chk2 phosphorylation in these subcellular fractions, we determined if Chk2 is a substrate for DNA-PK. Purified DNA-PK holoenzyme phosphorylated wild-type or kinase-defective Chk2 at Thr68 most efficiently in the presence of both ATP and DNA (Fig. 2A, lanes 3 and 6). We next determined if fractions from DNA-PK-defective cells would also phosphorylate Chk2. M059J is a human glioma cell line that does not express DNA-PKcs, and is the only DNA-PKcs-defective cell line of human origin. M059K is a cell line with wild-type DNA-PKcs that was established from the same malignant glioma and has DNA-PK activity (38). Subcellular fractions were prepared from M059J and M059K cells, as well as from NBS and HCC1937 cell lines which have mutated NBS1 and BRCA1, respectively. Hypophosphorylated Chk2 produced by translation in wheat germ extracts was not efficiently phosphorylated at Thr68 by fractions from DNA-PK-negative M059J cells, while it was phosphorylated by fractions from M059K cells (Fig. 2B, compare lanes 11 and 12 to lanes 8 and 9), as well as by fractions from cells with mutated NBS1, BRCA1 (Fig. 2B, lanes 1–6), or ATM (Fig. 1B, lane 1). The protein levels of cytoplasmic (S1), soluble nuclear (S2), and chromatin-enriched (P) fractions from M059K and M059J cells were normalized by blotting for Grb2, Sp1, and histone 3, respectively (Fig. 2B, lanes 13–18). Cell lines V3-H15 and V3-KA4 were derived from the same parent clone of the DNA-PK-defective CHO cell line V3 by transfection with genes encoding wild type or kinase-defective DNA-PKcs, respectively. Reconstitution of DNA-PKcs expression in V3 cells complemented the radiosensitivity, but introduction of kinase-defective DNA-PKcs did not (15). Fractions from V3-H15 cells phosphorylated hypophosphorylated Chk2 much more efficiently than did fractions from V3-KA4 cells (Fig. 2C, compare lanes 1–3 to lanes 4–6). The data further suggest that in this in vitro assay, DNA-PK is the major kinase that phosphorylates Chk2, and that the phosphorylation is not dependent on NBS1 and Brca1.

The Interaction of Chk2 and Ku—The above data indicated a relationship between DNA-PK activity and Chk2 phosphorylation, so we next tested whether Chk2 and DNA-PK physically interact. In vitro transcribed-translated Chk2 was incubated with cellular extracts. Wild-type Chk2 communoprecipitated endogenous Ku70 and Ku80 (Fig. 3A, lanes 5 and 6), whereas Chk2 with defective kinase activity or a deleted FHA domain only interacted weakly with Ku (Fig. 3A, lanes 7–10). Chk2 binding to
Ku was unaffected by DNA damage (Fig. 3A, lanes 5 and 6). To further evaluate the specificity of the interaction between Chk2 and Ku, the interaction of endogenous proteins was studied (Fig. 3B, lanes 3 and 4). Chk2 and Ku70/Ku80 still co-precipitated in the presence of ethidium bromide (EtBr) (Fig. 3B, lanes 7 and 8), suggesting that the interaction is not mediated by DNA. Although these associations between Chk2 and the Ku complex may be indirect, bacterially expressed GST-Chk2 did pull down Ku from purified DNA-PK (data not shown).

**DNA Damage-induced Chk2 Phosphorylation in Vivo Is Reduced in DNA-PK-defective Cells**—We next determined whether cells lacking DNA-PK are impaired for Chk2 regulation. Phosphorylation and activation of Chk2 by upstream kinases such as PIKKs reduces Chk2 electrophoretic mobility (6, 39). When V3-H15 (with wild-type DNA-PKcs), or V3-KA4 (with kinase-defective DNA-PKcs) were irradiated, forms of Chk2 that comigrated with anti-FLAG beads. pCDNA-FLAG vector (Vec) was used for in vitro transcription-translation and subsequent immunoprecipitation with anti-FLAG beads as negative control. B, endogenous Chk2 and Ku70/Ku80 coimmunoprecipitate. MCF-7 cells were mock-irradiated or exposed to IR (10 Gy) and lysed with NETN buffer 30 min after irradiation. Chk2 was immunoprecipitated with rabbit polyclonal antibodies in the presence or absence of 200 μg/ml ethidium bromide (EtBr). Immunoblotting of equal immunoprecipitates was performed with anti-Ku70 and anti-Ku80 antibodies. The membrane was stripped and then reprobed with anti-FLAG or anti-Chk2 antibody. **IP,** immunoprecipitate; **IB,** immunoblot.

**Fig. 2.** Fractions from DNA-PK-defective cells do not phosphorylate Chk2 efficiently in vitro. **A,** DNA-PK phosphorylates Chk2 at Thr<sup>68</sup> in vitro. FLAG-Chk2 was produced by coupled transcription-translation in wheat germ extract and immunoprecipitated with anti-FLAG affinity beads. The immunoprecipitated wild type or kinase-defective Flag-Chk2 was incubated for 1 h at 30 °C with purified DNA-PK, in the absence or presence of ATP or 40 μg/ml sonicated salmon sperm DNA. **B** and **C,** in vitro translation products were incubated first for 1 h at 30 °C with either kinase buffer A only or cell fractions in kinase buffer A, in the presence of an ATP-regenerating system. Then FLAG-Chk2 was immunoprecipitated. **B,** fractions from DNA-PK-defective cells do not phosphorylate Chk2 efficiently in vitro. In vitro translated FLAG-Chk2 was incubated with fractions from NBS (with defective NBS1), HCC1937 (with defective BRCA1), M059K (DNA-PK<sub>cs</sub>-positive), or M059J (DNA-PK<sub>cs</sub>-negative) cells. Grb2, Sp1, and histone 3 (H3) were immunoblotted to normalize protein levels in the S1, S2, and P fractions of M059K and M059J cells. C, fractions from cells with kinase-defective DNA-PK<sub>cs</sub> do not phosphorylate Chk2 efficiently in vitro. In vitro translated FLAG-Chk2 was incubated with fractions from V3-H15 (V3 with wild-type DNA-PK<sub>cs</sub>) or V3-KA4 (V3 with kinase-defective DNA-PK<sub>cs</sub>) cells in kinase buffer A, or incubated with kinase buffer A only. Grb2 was blotted to normalize the protein level in the S1 fractions of V3-H15 and V3-KA4 cells. Nonspecific bands (ns) are shown to indicate the comparative protein levels in S2 and P fractions of V3-H15 and V3-KA4 cells in the immunoblots of either anti-Ku80 or anti-Or2 (the anti-Ku80 and anti-Or2 antibodies do not recognize hamster antigen). Phosphorylation of Thr<sup>68</sup> was monitored by immunoblotting with anti-α-Thr<sup>68</sup>-Chk2 antibodies. The membrane was stripped and then reprobed with anti-FLAG-HRP. **IB,** immunoblot; **S1,** cytosolic fraction; **S2,** soluble nuclear fraction; **P,** chromatin-enriched fraction.

**Fig. 3.** The interaction of Chk2 and Ku. **A,** in vitro translated Chk2 coimmunoprecipitates endogenous Ku70 and Ku80 in cellular lysates. U2-OS cells were mock-irradiated or exposed to IR (10 Gy) and lysed with NETN buffer 1 h after irradiation. For immunoprecipitation, in vitro translated FLAG-Chk2, wild-type, kinase-defective, or FHA-deleted, was mixed with U2-OS lysates, and FLAG-Chk2 was immunoprecipitated with anti-FLAG beads. pCDNA-FLAG vector (Vec) was used for in vitro transcription-translation and subsequent immunoprecipitation with anti-FLAG beads as negative control. B, endogenous Chk2 and Ku70/Ku80 coimmunoprecipitate. MCF-7 cells were mock-irradiated or exposed to IR (10 Gy) and lysed with NETN buffer 30 min after irradiation. Chk2 was immunoprecipitated with rabbit polyclonal antibodies in the presence or absence of 200 μg/ml ethidium bromide (EtBr). Immunoblotting of equal immunoprecipitates was performed with anti-Ku70 and anti-Ku80 antibodies. The membrane was stripped and then reprobed with anti-FLAG or anti-Chk2 antibody. **IP,** immunoprecipitate; **IB,** immunoblot.
between 2 and 20 Gy (Fig. 4A, compare lanes 1–9 to lanes 10–18). Similarly, IR enhanced Chk2 autophosphorylation activity to a greater extent in V3-H15 cells than in V3-KA5 cells (Fig. 4B, compare lanes 2 and 4).

We extended the in vivo analysis of Chk2 activation using human M059J cells with defective DNA-PK and M059K cells with wild-type DNA-PK. Besides the difference in expression level of DNA-PKcs, M059J also has lower levels of ATM (40, 41). Nonetheless, the radiosensitivity of M059J can be complemented by introducing a fragment of chromosome 8 harboring M059J cells was attenuated compared with M059K (Fig. 5A, upper panel, compare alternate lanes). This was consistent with the sustained Thr<sup>68</sup> phosphorylation of only non-shifted Chk2 in M059J, in contrast to the presence of phospho-Thr<sup>68</sup> of shifted Chk2 in M059K (Fig. 5A, bottom panel, compare alternate lanes).

Chk2 mobility shift induced by camptothecin, topoisomerase I inhibitor, was diminished in M059J compared with M059K at all time points (Fig. 5B, upper panel, compare lanes 7–12 to lanes 1–6). This was accompanied by a sustained Thr<sup>68</sup> phosphorylation of non-shifted Chk2 (Fig. 5B, middle panel, compare lanes 7–12 to lanes 1–6), and reduced phosphorylation at Chk2 Thr<sup>383/Thr<sup>387</sup></sup> (Fig. 5B, bottom panel, compare lanes 7–12 to lanes 1–6), the autophosphorylation sites in the activation loop of the kinase domain, which probably marks kinase-active Chk2 (13). Phosphorylation of Thr<sup>68</sup> is not sufficient for activation of Chk2, which is typically mobility-shifted in its active forms (11). These results suggest that DNA-PK plays a role in camptothecin-induced activation of Chk2. Some basal Chk2 Thr<sup>68</sup> or Thr<sup>383/Thr<sup>387</sup></sup> phosphorylation in the absence of exogenous DNA damage was observed in both M059K and M059J cells (Fig. 5B, middle and bottom panels, lanes 1 and 7), which may reflect endogenous DNA damage associated with the aberrant karyotypes and phenotypes of these cell lines (43).

siRNA directed against DNA-PK<sub>cs</sub> was used as another approach to further investigate the relationship of DNA-PK and Chk2 regulation. Around 48 h after transfecting U2-OS cells with siRNA targeting DNA-PK<sub>cs</sub> cells were irradiated with different doses of IR and allowed to recover for indicated lengths of time. Cells were lysed with SDS-PAGE sample buffer. Then lysates were subjected to immunoblotting with anti-Chk2 antibodies. Chk2, p-Chk2, pp-Chk2: hypophosphorylated or hyperphosphorylated Chk2. After IR. Cells were treated with 10 Gy IR and recovered for 1 h. Endogenous Chk2 was immunoprecipitated from detergent extracts and in vitro kinase assay was performed in the presence of [γ<sup>32</sup>P]ATP to study Chk2 autophosphorylation. H15, V3-H15 cells; KA4, V3-KA4 cells; IP, immunoprecipitation; IB, immunoblot.

**Fig. 4.** DNA damage-induced Chk2 activation is reduced in CHO cells with kinase-defective DNA-PK. A, IR-induced Chk2 phosphorylation in vivo is attenuated in V3-KA4 (DNA-PK<sub>cs</sub>-negative V3 with reintroduced kinase-defective DNA-PK<sub>cs</sub>) compared with V3-H15 (V3 with re-introduced wild-type DNA-PK<sub>cs</sub>) cells. Cells were treated with different doses of IR and allowed to recover for indicated lengths of time. Cells were lysed with SDS-PAGE sample buffer. Then lysates were subjected to immunoblotting with anti-Chk2 antibodies. Chk2, p-Chk2, pp-Chk2: hypophosphorylated or hyperphosphorylated Chk2. B, Chk2 kinase activity was increased to a greater extent in V3-H15 cells than in V3-KA4 cells after IR. Cells were treated with 10 Gy IR and recovered for 1 h. Endogenous Chk2 was immunoprecipitated from detergent extracts and in vitro kinase assay was performed in the presence of [γ<sup>32</sup>P]ATP to study Chk2 autophosphorylation. H15, V3-H15 cells; KA4, V3-KA4 cells; IP, immunoprecipitation; IB, immunoblot.
no obvious effect on Chk2 phosphorylation after IR (46). To determine if mTOR regulates Chk2, cells were pretreated with the mTOR inhibitor rapamycin before irradiation. Rapamycin had no effect on IR-induced Chk2 phosphorylation at Thr68 in both 293 cells and AT cells, but increased the electrophoretic mobility of the mTOR effector p70 S6 kinase (consistent with reduced phosphorylation) (Fig. 7C). Hence, DNA-PK is most likely to mediate the wortmannin-sensitive kinase activity detected in these assays.

**DISCUSSION**

We have presented several lines of evidence indicating that, besides ATM and ATR, DNA-PK is an additional kinase that phosphorylates and activates Chk2. A wortmannin-sensitive kinase activity in subcellular fractions catalyzed phosphorylation of Chk2. This activity is present in fractions from ATM-deficient cells. DNA, which activates DNA-PK, enhanced the Chk2 phosphorylation. Purified DNA-PK phosphorylated Chk2 at the biologically important Thr68 site. Fractions from DNA-PK-defective cell lines did not phosphorylate Chk2 efficiently. Endogenous Chk2 interacted with Ku70 and Ku80. Damage-induced Chk2 phosphorylation and kinase activity were attenuated in cells with defective DNA-PK. Down-regulation of DNA-PK by either siRNA or the inhibitor NU7026 reduced Chk2 phosphorylation after DNA damage. Simultaneous knockdown of ATM and DNA-PK further reduced Chk2 Thr68 phosphorylation comparing to cells with ATM knockdown. Finally, IR-induced Chk2 phosphorylation was still sensitive to wortmannin in cells with low levels of functional ATM and ATR, suggesting that other PIKKs, including DNA-PK, participate in Chk2 phosphorylation after DNA damage.

The enhancement of *in vitro* Chk2 phosphorylation by the addition of DNA (Fig. 1C) strongly suggests a role for DNA-PK, which is the major PIKK activated by DNA (14, 16). Biochemical analysis and electron microscopy both indicate that ATM and ATR are capable of interacting directly with DNA (47–49). However, there is no consensus on the effects of DNA on ATM and ATR activity. Some groups report that DNA activates ATM (41, 47) or ATR (36, 37). But, other groups did not observe a stimulatory effect of DNA on the kinase activity of ATM or ATR (35, 50, 51). Some recent findings have further excluded a role of DNA in ATM activation (2, 52). The phosphorylation of Chk2 *in vitro* by ATM purified from human placenta was not enhanced by the addition of DNA (34). Moreover, the finding that fractions from DNA-PK-defective cell lines did not phosphorylate Chk2 efficiently *in vitro* further substantiated the role of DNA-PK in the *in vitro* Chk2 phosphorylation assay (Fig. 2, B and C).

The observation that Chk2 interacts with Ku70/Ku80 constitutively suggests that Chk2 may be recruited to DNA-PKcs through an interaction with the Ku heterodimer. The failure to consistently detect DNA-PKcs in these complexes (data not shown) may reflect a transient association, comparable to the lack of stable association of Chk2 with ATM, or the dissociation of DNA-PKcs from a Ku-DNA complex after DNA-PKcs activation.

Chk2 with defective kinase activity or a deleted FHA domain showed attenuated binding with Ku (Fig. 3A), suggesting that Chk2 kinase activity and its FHA domain are involved in the recruitment of Chk2 to Ku. Since Ku is phosphorylated *in vivo* (53) and *in vitro* (54), this may indicate a mediator-like function of phospho-Ku in recruiting Chk2 to sites of DNA damage. This would be similar to a mechanism postulated for a phosphorylated mediator, budding yeast Rad9, in binding yeast Chk2 (Rad53) (55).

Mounting evidence indicates that kinases other than ATM are involved in transducing damage checkpoint signaling to Chk2. For example, Chk2 can be phosphorylated and activated independent of ATM after high levels of IR, HU, or UV (6, 39). In ATM−/− lymphoblasts or fibroblasts, Chk2 can still be activated by some other wortmannin- and caffeine-sensitive kinase(s) after irradiation (56). Studies of Chk2−/− mice suggest that Chk2 regulates p53-dependent apoptosis via both ATM-dependent and ATM-independent mechanisms (57). In response to DNA DSBs, ATM plays a major role during the immediate, rapid phase, while ATR participates later to maintain the damage response (1, 58). Recent studies of conditional knockout ATM flox−/− or/and ATM−/− MEF cells indicate that ATM and ATR both contribute to the early phase of G2/M arrest after IR, while ATR acts as the major kinase regulating the late
phase of G2/M arrest. In contrast, G2/M arrest induced by aphidicolin, a DNA polymerase inhibitor, is intact in ATR and ATM double knockout MEF cells, suggesting the role of another upstream kinase (59). Similarly, some topoisomerase II inhibitors, such as etoposide and adriamycin, might activate Chk2 in an ATM/ATR-independent manner (60). Our findings suggest a partially redundant role of DNA-PK in activating Chk2, because Chk2 activation was only attenuated or delayed in DNA-PK-defective cells (Figs. 4, 5, and 6, A and B).

Depletion of ATR by siRNA in AT cells did not significantly affect Chk2 phosphorylation by IR treatment, indicating that Chk2 activation is not dependent on ATR under our experimental conditions. This agrees with studies of MEFs with conditional knockout of ATR (59). In the absence of both ATM and ATR, Chk2 phosphorylation after IR was still inhibited by wortmannin (Fig. 7A), suggesting that other wortmannin-sensitive PIKKs, such as DNA-PK, hSMG-1, or mTOR, participate in phosphorylating Chk2. DNA-PK stands out as a strong candidate in this assay, since hSMG-1 down-regulation by siRNA had no obvious effect on IR-induced Chk2 phosphorylation (46). mTOR is mainly involved in positive regulation of protein synthesis, cell growth, and proliferation. The pretreatment of cells with rapamycin, a specific inhibitor of mTOR, has no effect on IR-induced Chk2 phosphorylation (Fig. 7C), suggesting mTOR does not play a role in regulating Chk2 under our experimental conditions. Other recent reports are consistent with the in-

![Figure](http://www.jbc.org/)

**FIG. 6.** Down-regulation of DNA-PK leads to reduced Chk2 phosphorylation after IR. A, DNA-PKcs knockdown by siRNA in U2-OS cells attenuates IR-induced Chk2 phosphorylation. The luciferase GL2 siRNA was used as control (c). Two days post-transfection, cells were irradiated and lysed with SDS-PAGE sample buffer after the different recovery time indicated. Ku70 was blotted as loading control. B, simultaneous knockdown of ATM and DNA-PKcs by siRNA in U2-OS cells. The luciferase GL2 siRNA was used as control (c). ATM siRNA; A + P, simultaneous ATM and DNA-PKcs siRNA. Ku80 was blotted as loading control. C, inhibition of DNA-PKcs by NU7026 in AT cells (A-T22JE-T) attenuates IR-induced Chk2 phosphorylation. Cells were pretreated with NU7026 or Me2SO for 1 h before IR. Cells were irradiated and lysed with SDS-PAGE sample buffer. Phosphorylation of Thr68 was monitored by immunoblotting with anti-pThr68-Chk2 antibodies. The membrane was stripped and then reprobed with anti-Chk2.
involvement of DNA-PK in the phosphorylation and activation of Chk2. Purified DNA-PK phosphorylates an N-terminal fragment of GST-Chk2 (amino acids 1–222), and this activity was increased dramatically in the presence of DNA (34). Point mutation studies showed that DNA-PK preferentially phosphorylates GST-Chk2 (amino acids 1–92) at Thr68 in vitro (4). The kinase activity of Chk2 immune complexes prepared from DNA-PK-deficient M059J cells increased less post-IR than the activity from M059K cells (61). Studies of ATM−/−, DNA-PKcs−/−, Chk2−/− or p53−/− MEF cells expressing E1A suggest that Chk2 is involved in latent p53-mediated apoptosis, which is independent of ATM, but requires DNA-PK. DNA-PKcs−/− cells showed an apoptosis deficiency comparable to Chk2−/− cells (20, 62). Both DNA-PK and Chk2 are required to activate p53 DNA binding activity in vitro, but DNA-PK does not act upstream of Chk2 in this assay. ATM is not required for the activation of p53 by DNA-PK and Chk2 (63).

Some reports seem to contradict a role for DNA-PK in acti-
tion of Chk2. IR-induced Thr68 phosphorylation of Chk2 in GM00558 lymphoma cells was not affected significantly after treating cells with vanillin, a recently characterized DNA-PK inhibitor (64). The Thr68 phosphorylation of Chk2 was comparable in M059K and M059J cells after 5 Gy of irradiation (63). The seeming discrepancies between these studies and ours could be explained by the different involvement of PIKKs in different cell types, as well as differences in experimental format. Besides ATM and ATR, DNA-PK is emerging as another important upstream PIKK in DNA damage signaling. H2AX is rapidly phosphorylated by ATM and DNA-PK jointly after IR, and is a central regulator of ionizing radiation-induced foci (IRIF) (65). γH2AX (phosphorylated H2AX) is essential for the retention of checkpoint mediators, including BRCT domain-containing 53BP1, Brca1, and MDC1/NFBD1, at the damage sites (66–68). 53BP1, Brca1, or MDC1/NFBD1 regulates Chk2 phosphorylation after DNA damage (69–71). DNA-PKcs autophosphorylation (IRIF) colocalize with γH2AX, 53BP1, and MDC1/NFBD1 foci (72, 73). On the other hand, MDC1/NFBD1 associates with the candidate Holliday junction resolvase Mus81, a Chk2 substrate (74). Chk2 interacts with MSH2, one of the mismatch repair proteins (75). Chk2 also phosphorylates Brca1, an event that induces the release of Brca1 from Chk2, and that is required for Brca1-dependent regulation of both HR (homologous recombination) and NHEJ (7). Besides ATM and ATR, DNA-PK is emerging as another important upstream PIKK in DNA damage signaling. H2AX is rapidly phosphorylated by ATM and DNA-PK jointly after IR, and is a central regulator of ionizing radiation-induced foci (IRIF) (65). γH2AX (phosphorylated H2AX) is essential for the retention of checkpoint mediators, including BRCT domain-containing 53BP1, Brca1, and MDC1/NFBD1, at the damage sites (66–68). 53BP1, Brca1, or MDC1/NFBD1 regulates Chk2 phosphorylation after DNA damage (69–71). DNA-PKcs autophosphorylation (IRIF) colocalize with γH2AX, 53BP1, and MDC1/NFBD1 foci (72, 73). On the other hand, MDC1/NFBD1 associates with DNA-PK/Ku complex and regulates DNA-PKcs autophosphorylation (73).

In addition to delaying cell cycle progression and inducing apoptosis upon DNA damage, Chk2 also plays vital role in the regulation of DNA repair. Phosphorylation of p53 at Ser20 by Chk2 stabilizes p53, which enhances its transcriptional ability to increase DNA repair (7). Through its FHA domain, Chk2 associates with the candidate Holliday junction resolvase Mus81, a Chk2 substrate (74). Chk2 interacts with MSH2, one of the mismatch repair proteins (75). Chk2 also phosphorylates Brca1, an event that induces the release of Brca1 from Chk2, and that is required for Brca1-dependent regulation of both HR (homologous recombination) and NHEJ (7). Besides ATM and ATR, DNA-PK is emerging as another important upstream PIKK in DNA damage signaling. H2AX is rapidly phosphorylated by ATM and DNA-PK jointly after IR, and is a central regulator of ionizing radiation-induced foci (IRIF) (65). γH2AX (phosphorylated H2AX) is essential for the retention of checkpoint mediators, including BRCT domain-containing 53BP1, Brca1, and MDC1/NFBD1, at the damage sites (66–68). 53BP1, Brca1, or MDC1/NFBD1 regulates Chk2 phosphorylation after DNA damage (69–71). DNA-PKcs autophosphorylation (IRIF) colocalize with γH2AX, 53BP1, and MDC1/NFBD1 foci (72, 73). On the other hand, MDC1/NFBD1 associates with DNA-PK/Ku complex and regulates DNA-PKcs autophosphorylation (73).

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