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The integrity of chromosomal DNA is continuously monitored throughout the cell division cycle. DNA damage activates signaling pathways called checkpoints that induce cell cycle arrest or apoptosis. Defects in checkpoint control can result in genomic instability and cancer predisposition (15). The Chk2 protein kinase is a key mediator of the DNA damage checkpoint that responds to DNA double-strand breaks (DSBs) (7, 8, 31). DNA DSBs induce the activation of ATM (ataxia telangiectasia mutated), which subsequently phosphorylates Chk2 on T68 within the N-terminal SQ/TQ cluster domain (3, 32, 33). T68 phosphorylation is required for efficient oligomerization and subsequent auto-/transphosphorylation of Chk2 at other regulatory sites, including T379, S456, and S516 (2, 22, 41, 53).

Heterozygous germ line mutations in CHK2 are associated with a p53-independent variant form of the Li-Fraumeni heritable cancer predisposition syndrome (6). Cells derived from Chk2-deficient mice exhibit defects in their ability to delay entry into S phase, sustain a G2 cell cycle arrest, and undergo apoptosis in response to DNA damage (16, 46). These effects have been attributed to a failure of p53 to be properly modified in Chk2-deficient cells. Chk2 phosphorylates p53 on S20 in vitro. S20 phosphorylation has been proposed to mediate p53 stabilization by preventing interaction of p53 with its negative regulator MDM2; however, the exact function for this phosphorylation event remains unclear (9, 10, 17, 20, 42, 52). In addition to p53, additional substrates of Chk2 include Brca1, MDMX2, PML, p73, and E2F1 (11, 26, 37, 45, 47, 54).

The Chk2 protein kinase protects genome integrity by promoting cell cycle arrest or apoptosis in response to DNA double-strand breaks, and Chk2 mutations are found in both familial and sporadic cancers. Exposure of cells to ionizing radiation (IR) or radiomimetic drugs induces Chk2 phosphorylation by ATM, followed by Chk2 oligomerization, auto-/transphosphorylation, and activation. Here we demonstrate that Chk2 is ubiquitinated upon activation and that this requires Chk2 kinase activity. Serine 379 (S379) was identified as a novel IR-inducible autoprophosphorylation site required for ubiquitination of Chk2 by a Cullin 1-containing E3 ligase complex. Importantly, S379 was required for Chk2 to induce apoptosis in cells with DNA double-strand breaks. Thus, auto-/transphosphorylation of S379 is required for Chk2 ubiquitination and effector function.

The covalent modification of cellular proteins by ubiquitin is best known for its role in mediating their proteasome-dependent proteolysis. However, recent studies have demonstrated that ubiquitination can also modify the enzymatic activity, trafficking, localization, and binding partners of the modified protein (21, 40). Here we show that Chk2 phosphorylates itself on S379 in response to DNA damage. This in turn, facilitates Chk2 ubiquitination by an E3 ligase complex containing Cullin 1 (Cul 1). Ubiquitination of Chk2 did not detectably alter its stability. Importantly, S379 was shown to play an important role in the ability of Chk2 to induce apoptosis in cells with DNA DSBs. Thus, ubiquitination of Chk2 may regulate Chk2 effector function.

MATERIALS AND METHODS

Antibodies and Western blotting. Endogenous Chk2 was detected either with a mouse monoclonal antibody (Upstate, clone 7) or with a rabbit polyclonal antibody raised against bacterially produced glutathione S-transferase (GST)-Chk2 (14). Flag-Chk2 fusion proteins were precipitated with anti-Flag M2 antibody-agarose affinity gel (Sigma Chemical Co.) and detected by Western blotting with anti-Flag M2 monoclonal antibody (Sigma Chemical Co.). Myc-Chk2 fusion proteins were immunoprecipitated with anti-Myc mouse monoclonal 9E10-conjugated aga­rose (Santa Cruz Biotechnology) and detected by Western blotting with anti-Myc A14 rabbit polyclonal antibody (Santa Cruz Biotechnology). Bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse (ICN/CAPPEL), HRP-goat anti-rabbit (Zymed) secondary antibodies, and proteins were visualized using ECL (GE Healthcare Life Sciences). Antibodies specific for Chk2 phosphorylated on S379 were generated by immunization of rabbits with the phosphopeptide CILGET-pS-LMR coupled to keyhole limpet hemocyanin. Antibodies specific for Chk2 phosphorylated on T68, T383/387, and S516 have been described previously (41). Ubiquitination assays carried out in vitro were probed with a ubiquitin-specific antibody (P4D1; Santa Cruz Biotechnology).

Cell culture and treatment of cells with DNA-damaging agents. HEK-293 and U2OS cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine growth serum, 100 units/ml (each) penicillin and streptomycin, and 1 mM L-glutamine (complete DMEM). Chk2 null mouse embryo fibroblasts (MEFs) (16) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, 2 mM L-glutamine, and 0.1 mM nonessential amino acids. In some cases, cells were exposed to 10 Gy from a 60Co source or were treated with complete DMEM containing 20 μM etoposide (VP16; Sigma Chemical Company).
Plasmids, pET15b-Chk2-WT (wild type) and -D368N, pcDNA3-Myc-Chk2-WT and -D368N, and pcDNA3-Flag-Chk2-WT, -D368N,-T68A, -T383/387A, and -S16A have been described previously (41). pET15b-Chk2-S379A was generated by site-directed mutagenesis of pET15b-Chk2-WT using the QuikChange site-directed mutagenesis kit (Stratagene) and the primers 5′-TCAAGGAGAGCCTCTCTAGAGACCCTCC-3′ (forward) and 5′-GGTTCACATAAGGTCTCTAAGGAGAGCCTCTCC-3′ (reverse). The same primers were used to generate pcDNA3-Flag-Chk2-WT and pcDNA3-Myc-Chk2-S379A by site-directed mutagenesis of pcDNA3-Flag-Chk2-WT encoding Myc-Chk2 into the corresponding sites of pcDNA3/FRT (Invitrogen). pcDNA5/FRT-Myc-Chk2-D368N and pcDNA5/FRT-Myc-Chk2-S379A was generated by inserting the KpnI/XhoI fragment of pcDNA3-Myc-Chk2-WT encoding Myc-Chk2 into the corresponding sites of pcDNA3/FRT (Invitrogen). pcDNA5/FRT-Myc-Chk2-D368N was generated by inserting the KpnI/XhoI fragment of pcDNA3-Myc-Chk2-WT encoding Myc-Chk2 into the corresponding sites of pcDNA3/FRT (Invitrogen). pcDNA5/FRT-Myc-Chk2-S379A was generated in a similar manner. Sequences of all mutants were verified by DNA sequencing. Plasmids encoding Myc-tagged Cullin proteins and dominant-negative forms of Cul 1 and Cul 3 have been described previously (12, 36, 43). The wild-type and -D368N; and pcDNA3-Flag3-Chk2-WT, -D368N, -T68A, -T383/387A, and -S16A have been described previously (41). The wild-type and -D368N; and pcDNA3-Flag3-Chk2-WT, -D368N, -T68A, -T383/387A, and -S16A have been described previously (41).

Purification of bacterially produced Chk2 protein. Escherichia coli strain BL21 was transformed with pET15b plasmids encoding His6-Chk2-WT, His6-Chk2-D368N, and His6-Chk2-S379A. Cultures were grown at 37°C to an optical density at 600 nm of 0.5, at which time isopropyl-1-thio-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM and protein expression was induced at 37°C for 4 h. Bacterial pellets were resuspended in NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EGTA, 0.5% NP-40) supplemented with protease inhibitors (2 mM PMSF, 0.15 unit/ml of aprotinin, and 20 μM leupeptin), 1 μM microcystin, and 2 mM mg/lysozyme and then rocked at 4°C for 30 min. Cells were lysed by sonication (50% duty cycle for 20 bursts). Lysates were clarified by centrifugation (14,000 × g for 15 min). His6-tagged proteins were precipitated by incubation with Ni2+–nitrilotriacetic acid agarose (Qiagen) for 2 h at 4°C. Precipitated proteins were washed twice in lysis buffer and two lysis buffer without NP-40. Bound proteins were eluted in elution buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM DTT) containing 100 mM imidazole and dialyzed overnight at 4°C in dialysis buffer (50 mM Tris-HCl [pH 7.4], 2 mM DTT). Purified His-Chk2 proteins were concentrated using Vivaspin columns (Vivascience, Inc.).

Generation of U2OS/FRT stables lines. The Flp-In system (Invitrogen) was used in order to create U2OS cell lines that stably express Myc-tagged Chk2 (pFRT/Myc-Chk2). U2OS cells were transfected with Flp-In FRT-Myc-Chk2-WT, -Myc-Chk2-D368N, or -Myc-Chk2-S379A using Lipofectamine 2000 (Invitrogen), and transfected colonies were selected in medium containing 50 μg/ml Zeocin (Invitrogen). Single colonies were grown up, their β-galactosidase activity was measured, and Southern analysis was performed to determine the number of FLP recombination target (FRT) sites inserted into the genome. The clone selected, U2OS/FRT16, had only one FRT site inserted into the genome. The clone was then cotransfected with pOG44, a vector for transient transfection of the Flp recombinase, and with pcDNA5/FRT-Myc3-Chk2-WT, -Myc-Chk2-D368N, or -Myc-Chk2-S379A using Lipofectamine 2000. The pcDNA5/FRT-Myc3-Chk2 vector contains the coding region for Chk2 (wild type or point mutants) and possesses an FRT site for homologous recombination. After insertion of the Chk2 gene into the FRT site, Chk2 transcription is driven by the human cytomegalovirus immediate-early enhancer/promoter. The pcDNA5/FRT vector also carries a hygromycin resistance gene to allow for selection of stable cells in 100 μg/ml hygromycin (Invitrogen). Homologous recombination of Myc3-Chk2 into the FRT site inactivates the lacZ-Zeo fusion gene, and stable cells are no longer Zeocin resistant. Hygromycin-resistant colonies were grown as pools in DMEM containing 10% fetal bovine serum, 100 μg/ml hygromycin, 100 units/ml (each) penicillin and streptomycin, and 1 mM l-glutamine.

Phosphorylation experiments with Chk2 null MEFs. Chk2 null MEFS were transfected with Flag-Chk2 (WT, D368N, or S379A) (44). At 48 h posttransfection, cells were harvested and lysed in MCLB. Lysates were immunoprecipitated with anti-Flag antibody. Densitometry analysis was performed using Image J software (1).
FIG. 1. Chk2 autophosphorylation on S379 regulates ubiquitination. (A) HEK-293 cells were transfected with plasmids encoding Flag3-Chk2 (WT and D368N) and Myc-tagged ubiquitin. At 42 h posttransfection, cells were treated with MG132 for 2 h, and Chk2 proteins were immunoprecipitated using an antibody specific for the Flag epitope. Ubiquitinated proteins were visualized by immunoblotting with antibodies specific for either Myc (top left panel) or Chk2 (top right panel). Precipitated Flag3-Chk2 was detected with antibodies specific for Flag (bottom left panel) or Chk2 (bottom right panel). (B to D) Asynchronously growing HEK-293 cells expressing Flag3-Chk2-WT (B), Flag3-Chk2-D368N (C), or Flag3-Chk2-S379A (D) were incubated with 32P-labeled inorganic phosphate in the presence of 25 μM VP16. Radiolabeled Chk2 proteins were digested with trypsin, and peptides were subjected to two-dimensional phosphopeptide mapping. The arrow in panel D denotes the absence of a phosphopeptide that is present in the map of wild-type Chk2. (E) Alignment of sequences inclusive of and surrounding S379 in the indicated Chk2 orthologs. The asterisk denotes S379 in human Chk2 and conserved residues in other orthologs. (F) HEK-293 cells were transfected with plasmids encoding Flag3-Chk2 (WT, D368N, and S379A). Immunoprecipitation (IP) with Flag antibody followed by Western blot (IB) with Myc or Flag antibody.
20 μM etoposide for the indicated time periods. Cells were harvested at 24 and 48 h after treatment and fixed in 70% ethanol. Cells were then stained with 30 μg/ml of propidium iodide for 30 to 60 min and analyzed by flow cytometry using a Becton-Dickinson FACScan. The data were analyzed by using CELLQUEST software.

**RESULTS**

**Autophosphorylation of Chk2 on S379 is required for Chk2 ubiquitination.** Following DNA damage, Chk2 oligomerizes in a phosphorylation-dependent manner, a process that ultimately results in its full activation (2, 41, 53). Previously we demonstrated that Chk2 oligomerizes and auto/transphosphorylates in the absence of any exogenous DNA damage when expressed at high levels either in HEK-293 cells or in bacteria (41). As seen in Fig. 1A, a slower electrophoretic form of Chk2 was observed when wild-type (lane 1) but not kinase-inactive (lane 2) Chk2 was produced in HEK-293 cells, and this is due to Chk2 oligomerization followed by auto/transphosphorylation (41). Strikingly, wild-type but not kinase-inactive Chk2 was ubiquitinated when co-produced with Myc-tagged ubiquitin in these cells. This was demonstrated using antibodies that recognize either the tagged form of ubiquitin (Fig. 1A, left) or Chk2 itself (Fig. 1A, right). These results demonstrate that Chk2 ubiquitination requires Chk2 kinase activity.

To identify phosphorylation sites that might be required for Chk2 ubiquitination, HEK-293 cells were transfected with Flag-tagged Chk2 constructs, treated with etoposide (VP16), and radiolabeled with [32P]orthophosphate. The Flag-tagged proteins were immunoprecipitated and used to generate tryptic phosphopeptide maps. Wild-type Chk2 gave rise to seven resolvable phosphopeptides (labeled 1 to 7 in Fig. 1B). Interestingly, all seven were absent when 32P-labeled kinase-inactive Chk2 (Chk2-D368N) was subjected to two-dimensional phosphopeptide mapping (Fig. 1C). These results demonstrate that phosphopeptides 1 to 7 require the kinase activity of Chk2. The identity of individual phosphopeptides was determined by phosphoamino acid analysis and site-directed mutagenesis. Mutation of serine 379 to alanine (S379A) resulted in loss of phosphopeptide 3 (Fig. 1D), whereas mutation of serine 516 to alanine (S516A) resulted in loss of phosphopeptide 7 (data not shown). Since the NH2 terminus of Chk2 lacks trypsin cleavage sites from amino acid 3 to 95, phosphopeptides from this region are not resolvable using this approach and are represented on the two-dimensional maps as unresolved phosphopeptides along the x axis (Fig. 1B to D). S379 was also identified as a Chk2 autophosphorylation site by mass spectrometry (23). Interestingly, S379 is conserved in Chk2 orthologs from mice, flies, and budding yeast; a threonine residue is present in the equivalent site in the fission yeast Chk2 ortholog (Fig. 1E).

Ubiquitination assays were performed to determine if S379 was required for Chk2 ubiquitination. As seen in Fig. 1F, wild-type Chk2 was ubiquitinated in vivo (top panel, lane 1), and the kinase-inactive mutant (D368N) failed to be ubiquitinated (lane 2). Interestingly, the S379A Chk2 mutant also failed to be ubiquitinated in vivo (lane 3). In cells containing DNA DSBs, ATM initiates Chk2 activation by phosphorylating Chk2 on T68. T68 phosphorylation is required for efficient oligomerization and subsequent auto/transphosphorylation of Chk2 at other regulatory sites, including T383/387. As seen in Fig. 1F, both wild-type Chk2 and S379A, but not kinase-inactive Chk2, were phosphorylated on T383/387. This suggests that the S379A mutant of Chk2 retains kinase activity. This conclusion is further supported by the fact that the electrophoretic mobility of S379A is slower than that of kinase-inactive Chk2 (Fig. 1F, Flag−/Chk2 panel).

Several Chk2 mutants were also expressed in HEK-293 cells to determine requirements for Chk2 ubiquitination (Fig. 1G). Two kinase-inactive mutants (D368N and T383/387A) failed to be ubiquitinated (lanes 3 and 5), demonstrating the importance of Chk2 kinase activity for ubiquitination. Wild-type Chk2, T68A, and S516A are all kinase active, although the encoding Flag−/Chk2 (WT, D368N, and S379A) and Myc-tagged ubiquitin. At 48 h posttransfection, cells were lysed and Chk2 proteins were immunoprecipitated (IP) using an antibody specific for the Flag epitope. Ubiquitinated proteins were visualized by immunoblotting (IB) with an antibody to the Myc tag. The Myc blots were then stripped and probed with an antibody specific for the Flag tag. Phosphorylation of Chk2 on T383/387 and S379 was examined on total lysates using antibodies specific for these sites. (G) HEK-293 cells were transfected with expression plasmids encoding Flag−/Chk2 (WT, D368N, T68A, T383/387A, or S516A) and Myc-tagged ubiquitin (Myc-Ub). Chk2 proteins were immunoprecipitated using an antibody specific for the Flag epitope. Ubiquitinated proteins were visualized by immunoblotting with an antibody to the Myc tag. The Myc blots were then stripped and probed with an antibody specific for the Flag tag.
T68A mutant fails to be activated to the same extent as wild-type Chk2 and S516A in response to DNA damage (3, 32, 33, 41, 57). As seen in Fig. 1G, wild-type Chk2, T68A, and S516A were all ubiquitinated in vivo (lanes 2, 4, and 6), and the ubiquitination of T68A was less than that than observed for wild-type Chk2 and S516A. These findings underscore the importance of both kinase activity and S379 phosphorylation for Chk2 ubiquitination.

**Phosphorylation of S379 is IR inducible and requires Chk2 kinase activity.** To further characterize S379 phosphorylation, an antibody specific for Chk2 phosphorylated on this residue was generated (Fig. 1F and Fig. 2A). The antibody was then used to monitor endogenous Chk2 phosphorylation in vivo. As seen in Fig. 2B, endogenous Chk2 was inducibly phosphorylated on S379 within 30 minutes after IR treatment. Thus, S379 is a novel IR-inducible Chk2 phosphorylation site.

S379 is located within the activation loop of the Chk2 kinase domain. Thus, mutation of S379 could impair the kinase activity of Chk2. Therefore, S379A was tested for its ability to auto-/transphosphorylate in bacteria. As reported previously (41), overproduction of Chk2 in bacteria promotes Chk2 oligomerization and auto-/transphosphorylation (Fig. 2C, lane 1), whereas kinase-inactive Chk2 fails to be phosphorylated in bacteria (Fig. 2C, lane 2). Importantly, the S379A mutant displayed a shift in electrophoretic mobility and was phosphorylated on T68, T383/387, and S516 in bacteria (Fig. 2C, lane 3), demonstrating that mutation of S379 to alanine does not render Chk2 kinase inactive. The phosphorylation status of S379A was also monitored in CHO2 null MEFs (16) to eliminate any interactions between S379A and endogenous Chk2. As seen in Fig. 2D, wild-type Chk2 was phosphorylated on T68, T383/387, S379, and S516 (lane 1). Kinase-inactive Chk2 was phosphorylated on T68 but not on residues that require Chk2 kinase activity (T383/387 and S516). The finding that kinase-inactive Chk2 was not detectably phosphorylated on S379 demonstrates that phosphorylation of S379, like that of S16 and T383/387 but unlike that of T68, requires Chk2 kinase activity (Fig. 1F and 2D, lane 2). Notably, S379A was phosphorylated on T68, T383/387, and S516 in Chk2 null MEFs, demonstrating that S379A retains kinase activity in vivo (Fig. 2D, lane 3).

Next, kinase assays were performed to measure the activation of S379A following exposure of cells to IR (Fig. 2E). The kinase activity measured for S379A was similar to that of wild-type Chk2 in the absence of IR. IR induced an approximate 2.3-fold activation in the kinase activity of wild-type Chk2, whereas an approximate 1.5-fold activation in the kinase activity was measured for the S379A mutant. These results indicate that mutation of S379 does not inactivate the catalytic activity of Chk2 as does mutation of T383/387 but mutation of S379 does partially impair Chk2 activation by IR. Taken together, our results demonstrate that IR induces phosphorylation of Chk2 on S379, that S379 phosphorylation is dependent on Chk2 kinase activity, and that phosphorylation of S379 is required for Chk2 ubiquitination.

**Chk2 ubiquitination catalyzed by Cul 1-containing E3 ligase.** Next, experiments were performed to identify the E3 ubiquitin ligase responsible for Chk2 ubiquitination. Cullin-containing E3 ligases comprise the largest family of ubiquitin ligases in eukaryotic cells and consist of seven members (Cul 1, Cul 2, Cul 3, Cul 4A, Cul 4B, Cul 5, and Cul 7) (38). We first determined whether complexes between Chk2 and members of the Cullin family could be detected in vivo. As seen in Fig. 3A, cotransfection experiments revealed that Chk2 interacted with Cul 1, 3, and 4B (lanes 1, 3, and 5) but not with Cul 2, 4A, or 5 (lanes 2, 4, and 6). Next, dominant-negative forms of Cul 1, 3, and 4B were tested for their ability to block Chk2 ubiquitination in vivo (12, 36, 43). As seen in Fig. 3B, ubiquitination of wild-type Chk2 was blocked by a dominant-negative Cul 1 mutant (lane 4) but not dominant-negative forms of Cul 3 or 4B (lanes 5 and 6). As expected, kinase-inactive Chk2 (lanes 7 to 10) and S379A (lanes 11 to 14) were not ubiquitinated (Fig. 3B) and did not associate with Cul 1 (Fig. 4A, lanes 2 and 3) in vivo. Finally, assays were performed in vitro to monitor the ability of Cul 1-, 3-, and 4B-containing complexes to catalyze Chk2 ubiquitination. As seen in Fig. 4B, only Cul 1-containing complexes ubiquitinated Chk2 in vitro (lane 4). Neither kinase-inactive Chk2 (lanes 7 to 9) nor S379A (lanes 10 to 12) was ubiquitinated in vitro. These findings indicate that a Cul 1-containing E3 ligase is responsible for Chk2 ubiquitination in vivo.

**Role of Chk2 S379 phosphorylation.** Endogenous Chk2 protein levels were monitored in both the presence and absence of DNA DSBs in HEK-293 cells (Fig. 5A). In the presence of cycloheximide, Chk2 protein levels remained stable for at least 2 h in mock-irradiated cells (Fig. 5A, lanes 1 to 4 and 9 to 12). IR induces Chk2 phosphorylation, which, in turn, causes Chk2 to migrate more slowly on SDS gels (Fig. 5A, lanes 5 to 8). Quantitation of Western blotting results suggested that IR also promoted Chk2 turnover. However, when cell lysates were treated with λ phosphatase prior to Western blotting (Fig. 5A, lanes 13 to 16), Chk2 was observed to be stable in IR-treated cells. These results demonstrate that the Chk2 antibody used for Western blotting inefficiently recognizes phosphorylated forms of Chk2 and that the half-life of endogenous Chk2 is not dramatically altered in cells exposed to IR. Next, the half-life of ectopically expressed Chk2 was measured in HEK-293 cells, as wild-type Chk2 is ubiquitinated when overproduced in these cells (Fig. 1A and F). HEK-293 cells expressing wild-type and mutant forms of Flag-tagged Chk2 were incubated with cycloheximide, and Chk2 levels were monitored by Western blotting using an antibody specific to the Flag tag. As seen in Fig. 5B, levels of wild-type and mutant Chk2 proteins remained stable over the 4-h incubation period.

Protein ubiquitination regulates a plethora of biological activities in addition to proteosome-mediated degradation, including protein-protein interactions, enzymatic activity, and intracellular localization. Therefore, we tested whether S379 phosphorylation was required for Chk2 signaling. Chk2 is a critical regulator of DNA damage-induced apoptosis, particularly in response to agents that induce DNA DSBs (16, 19, 45, 46, 51, 54). To test whether S379 phosphorylation was required for Chk2-mediated apoptosis, U2OS cells stably expressing wild-type and mutant forms of Chk2 in the same genetic locus were generated. A FRT site was first introduced into U2OS cells to generate the U2OS/FRT parental line. Plasmids encoding Myc3-Chk2-WT, Myc3-Chk2-D368N, and Myc3-Chk2-S379A were then cotransfected with the Flp recombinase from *S. cerevisiae* to generate the U2OS/FRT stable cell lines expressing Myc3-Chk2 (WT and mutants) at the same locus. Densitometry analysis revealed that the Myc3-Chk2 proteins
FIG. 2. Phosphorylation and kinase activity of Chk2 S379A. (A) Lysates of HEK-293 cells expressing Flag-Chk2-WT or Flag-S379A were resolved by SDS-PAGE and blotted with an antibody specific for Chk2 phosphorylated on S379. The blot was then stripped and reprobed with an antibody specific for the Flag epitope. (B) Lysates from HEK-293 cells that had been mock irradiated (lane 1) or exposed to 10 Gy IR (lane 2) were resolved by SDS-PAGE and blotted with an antibody specific for Chk2 phosphorylated on S379 and an antibody specific for Chk2. (C) His6-Chk2-WT, -D368N, and -S379A were purified from bacteria. Matched amounts of protein were resolved by SDS-PAGE and blotted with the indicated antibodies. (D) Lysates from Chk2 null MEFs expressing Flag-Chk2-WT, -D368N, or -S379A were incubated with an antibody specific for the Flag epitope. Precipitates were resolved by SDS-PAGE and blotted with the indicated phospho-specific antibodies. The phospho-specific blots were then stripped and reprobed with an antibody specific for the Flag tag. (E) Myc3-tagged Chk2 proteins (WT and S379A) were precipitated from U2OS/FRT stable lines 30 min after mock irradiation (−) or exposure to 10 Gy IR (+). The precipitated Chk2 proteins were tested for their ability to phosphorylate GST-Cdc25C(200–256) in vitro. 32P-labeled GST-Cdc25C(200–256) was quantified using a Storm Imager. Error bars indicate the standard errors of the means for triplicate reactions performed for each Chk2 protein.
were expressed at approximately twofold the level of endogenous Chk2 in the U2OS/FRT lines (Fig. 6A).

Apoptosis was assayed by treating the U2OS/FRT stable cell lines with etoposide and analyzing DNA content by flow cytometry (45). As seen in Fig. 6B, cells expressing D368N or S379A were less susceptible to etoposide-induced apoptosis than were parental cells or cells expressing wild-type Chk2. These results demonstrate that both kinase activity and S379

![Diagram](http://mcmb.asm.org/)...
phosphorylation are required for the proapoptotic effector function of Chk2.

The phosphorylation status of endogenous Chk2 was monitored in each of the U2OS cell lines to determine whether D368N or S379A interfered with activation of endogenous Chk2. Chk2 activation can be determined indirectly by monitoring its mobility on SDS gels as well as by S516 phosphorylation. Part of the self-sustaining Chk2 activation cycle involves auto-/transphosphorylation of Chk2 on S516 as well as other residues, and this causes Chk2 to migrate more slowly on SDS gels. Phosphorylation of T68 is catalyzed by kinases in addition to Chk2 (3, 57), and as seen in Fig. 6C, IR induced the phosphorylation of endogenous Chk2 on T68 in each cell line (lanes 5 to 8). However, endogenous Chk2 failed to undergo mobility shifts (Fig. 6C, lane 7) and to be phosphorylated on S516 (Fig. 6D, lanes 8 and 9) in cells expressing D368N. This suggests that the kinase-inactive Chk2 mutant functions in a dominant-negative manner. In contrast, the activation and phosphorylation of endogenous Chk2 appeared normal in cells expressing S379A. Thus, expression of D368N blocked activation of endogenous Chk2, and this likely accounts for its ability to block DNA damage-induced apoptosis in U2OS cells. In contrast, results obtained for S379A indicate that this mutant interferes with pathways downstream of endogenous Chk2 activation.

DISCUSSION

In cells containing DNA DSBs, ATM initiates Chk2 activation by phosphorylating Chk2 on T68. This leads to Chk2 oligomerization, which facilitates Chk2 phosphorylation at additional sites through an auto-/transphosphorylation mechanism. Here we demonstrate that auto-/transphosphorylation of Chk2 on S379 also leads to its ubiquitination by a Cul 1-containing E3 ligase. To recruit specific substrates, Cul 1 binds the Skp1 adaptor protein (13, 44, 56). Skp1 then binds an F box protein, which in turn recruits substrates for ubiquitination. Additional studies will be required to identify the F box protein that targets Chk2 to the Cul 1 complex. In general, the recruit-
ment of substrates to the Skp1-Cul 1-F box complex requires that the substrate first be phosphorylated. In the case of Chk2, S379 phosphorylation is required for its interactions with Cul 1 and its subsequent ubiquitination. Mutants of Chk2 that failed to be phosphorylated on S379 (kinase-inactive Chk2 and the S379A mutant) did not associate with Cul 1 and were not ubiquitinated in vivo. This suggests that S379 phosphorylation may regulate interactions between the Cul-containing E3 ligase complex and Chk2. We were unable to detect significant changes in the half-life of Chk2 after its ubiquitination, consistent with studies reporting that Chk2 remains stable during a DNA damage checkpoint response (4, 5, 28, 54). Kass et al. recently reported that dephosphorylation on S456 is necessary for Chk2 ubiquitination and that ubiquitination regulates Chk2 turnover (22). Differences in results between these studies may be due to differences in the cell lines used or the type or intensity of DNA damage.

Although proteasome-dependent degradation of ubiquitinated proteins is the best-characterized function for this modification, there have been several additional functions reported for protein ubiquitination over the past few years (21, 39). In particular, both Brca1 and RNF8 are E3 ubiquitin ligases that translocate to sites of DNA damage and ubiquitinate proteins to recruit additional proteins to sites of damage and to activate signaling pathways (18, 24, 30, 49). It has not been determined when Chk2 becomes ubiquitinated. The adaptor protein MDC1 recruits RNF8 to damaged chromatin in a phosphorylation-dependent manner, and the ubiquitin-interacting motif-containing protein RAP80 mediates the localization of Brca1 to DNA damage foci (18, 30, 49, 50). MDC1 associates with phosphorylated Chk2 at foci following DNA damage and contributes to Chk2-mediated apoptosis (27). It will be important to determine if Chk2 ubiquitination recruits Chk2 to foci by, for example, binding a ubiquitin-interacting motif-containing protein such as RAP80 at sites of DNA damage. Alternatively, Chk2 may become ubiquitinated after its translocation to foci. Chk2 has also been shown to associate with USP28, a deubiquitinating enzyme, but the functional significance of this interaction requires further study (55).

The initial activation of Chk2 involves its translocation to
FIG. 6. Phosphorylation of S379 in activation of Chk2 and apoptosis induction. (A) U2OS/FRT parental cells were stably transfected with plasmids encoding Myc-Chk2-WT, Myc-Chk2-D368N, or Myc-Chk2-S379A as described in Materials and Methods. Lysates were blotted with an antibody against Chk2, and the relative expression level of Myc-Chk2 versus endogenous Chk2 for each cell line was quantified by chemiluminescence using a Storm Imager; the ratio of ectopic to endogenous Chk2 expression is indicated. (B) U2OS/FRT parental and Chk2-expressing cells were treated with etoposide for the indicated times. The percentage of cells containing sub-2N DNA content was assessed by propidium iodide staining and fluorescence-activated cell sorter analysis. Error bars denote standard errors of the means (n = 4). (C and D) U2OS/FRT parental and Chk2-expressing cells were exposed to IR. Lysates were prepared and Western blotting was performed with the indicated antibodies.
sites of DNA damage. However, this relocalization is transient and Chk2 becomes rapidly redistributed throughout the nucleus in order to carry out its signaling function (29). The fact that the S379A mutant was not ubiquitinated and was impaired in its ability to induce apoptosis in cells with DNA DSBs suggests that Chk2 ubiquitination and signaling may be coupled. Expression of a kinase-inactive mutant of Chk2 blocked phosphorylation of endogenous Chk2 and impaired apoptosis in cells with DNA DSBs. Thus, kinase-inactive Chk2 may function as a dominant-negative mutant by oligomerizing with endogenous Chk2 and thereby preventing subsequent auto- or transphosphorylation events. In contrast, the S379A mutant did not detectably alter endogenous Chk2 phosphorylation. This suggests that proximal Chk2 activation events (Chk2 oligomerization and auto- or transphosphorylation) were not per-

cision as a dominant-negative mutant by oligomerizing with en-


