Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G_2 checkpoints

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Checkpoint kinase (Chk)1 is an evolutionarily conserved protein kinase that was first identified in fission yeast as an essential component of the DNA damage checkpoint. In mice, Chk1 provides an essential function in the absence of environmentally imposed genotoxic stress. Here we show that human cells lacking Chk1 exhibit defects in both the ionizing radiation (IR)-induced S and G_2 checkpoints. In addition, loss of Chk1 resulted in the accumulation of a hypophosphorylated form of the Cdc25A protein phosphatase, and Chk1-deficient cells failed to degrade Cdc25A after IR. The IR-induced S and G_2 checkpoints were partially restored in Chk1-deficient cells when Cdc25A accumulation was interfered with. Finally, Cdc25A was phosphorylated by Chk1 *in vitro* on similar sites phosphorylated *in vivo*, including serine-123. These findings indicate that Chk1 directly phosphorylates Cdc25A during an unperturbed cell cycle, and that phosphorylation of Cdc25A by Chk1 is required for cells to delay cell cycle progression in response to double-strand DNA breaks.

Checkpoint kinase (Chk)1 was first identified in fission yeast as an essential component of the DNA damage checkpoint but was later shown to also function in the DNA replication checkpoint (1–7). Although *chk1* is not an essential gene in fission yeast, it is essential in mice (1, 3, 8, 9). The essential function provided by murine Chk1 throughout early embryonic development has not been determined. Mouse embryos and conditional embryonic stem cell lines lacking Chk1 exhibit defective checkpoint responses to replication blocks and DNA-damaging agents establishing a checkpoint function for Chk1 in mice (8, 9). Evidence that Chk1 contributes to G_2 checkpoint control in human cells comes from studies showing that agents such as UCN-01 and SB-218078, which are potent inhibitors of Chk1, abrogate G_2 checkpoint function in human cells (10–12). However, it is clear that Chk1 is not the only kinase targeted by UCN-01 (see below).

Although Chk1 is conserved throughout evolution, the signals that Chk1 responds to have diverged among eukaryotic organisms. In fission yeast, Chk1 responds to DNA damage induced by ionizing radiation (IR) and UV light (UV) (3, 4), as well as to DNA replication inhibitors (2, 5, 6). In *Xenopus* (*Xe*), Chk1 is activated by UV irradiation and by agents that block DNA replication (13). In contrast to fission yeast, *Xe* Chk1 is not activated by double-stranded DNA breaks of the type induced by IR (13). In *Xe* and humans, Chk1 is phosphorylated and activated by ATR (ATM and Rad3-related protein kinase), and the ATR/Chk1 pathway responds to agents that impair DNA replication, either directly (hydroxyurea, aphidicolin) or indirectly (UV irradiation) (8, 13, 14). Human Chk1 is phosphorylated on serines 317 and 345 by ATR *in vitro*, and these residues are phosphorylated *in vivo* in an ATR-dependent manner (8, 14).

The contribution made by Chk1 to the IR-induced DNA damage checkpoint in humans remains controversial. Some studies have reported that the electrophoretic mobility of human Chk1 is retarded after IR (8, 10, 15), whereas other studies have failed to observe changes in the electrophoretic mobility of Chk1 after IR treatment. Human Chk1 is phosphorylated on serine-345 after IR (8), and we consistently observe a 2-fold increase in Chk1 kinase activity after IR that also accompanies S317 phosphorylation (Fig. 7, which is published as supporting information on the PNAS web site, www.pnas.org). In humans, fission yeast, and *Xe*, Chk1 has been proposed to regulate the G_2 checkpoint by phosphorylating the Cdc25C protein phosphatase on residue(s) that facilitate the binding of 14-3-3 proteins (5, 15–18). In addition, human Chk1 has been proposed to regulate the stability of the Cdc25A protein phosphatase in UV-damaged cells (19).

In this study, we investigated the contribution made by human Chk1 to the cell division cycle both in the absence and presence of checkpoint activation. We report that cells deficient in human Chk1 exhibit radio-resistant DNA synthesis and do not delay in G_2 after IR treatment. We provide evidence that Chk1 directly regulates Cdc25A throughout an unperturbed cell cycle, and that the integrity of the Chk1/Cdc25A regulatory pathway is essential for cells to respond appropriately to ionizing radiation.

Materials and Methods

Cell Culture. HeLa cells were grown in DMEM (GIBCO) supplemented with 10% FBS/100 units/ml penicillin and streptomycin/1 mM glutamine (culture medium). Normal human diploid fibroblasts (American Type Culture Collection, WI 30) were grown in minimum essential medium containing 10% FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 units/ml penicillin and streptomycin.

Antibodies and Western Blotting. Chk1 was detected with monoclonal (G-4) or polyclonal (FL-476) antibodies (Santa Cruz Biotechnology). Chk2 was detected with a monoclonal antibody from Neomarkers (Fremont, CA). Endogenous Cdc25A was immunoprecipitated and immunoblotted with Ab-3 (Neomarkers). Human Cdc25C and Cdc25B were detected with antibodies sc-326 and -327, respectively (Santa Cruz Biotechnology), and a polyclonal antibody was used to detect C-TAK1 (20). A polyclonal antibody was used to immunoprecipitate human cyclin B1 (21). Phospho-histone H3 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Bound primary antibodies were detected with either horseradish peroxidase (HRP)-goat anti-mouse antibody (ICN/Jackson ImmunoResearch) or HRP-goat anti-rabbit antibody (Zymed), and proteins were visualized by chemiluminescence by using the enhanced chemiluminescence reagent (Amersham Pharmacia). Antibodies specific for human Cdc25A phosphorylated on serine-123 were generated by immunization of rabbits with the phosphopeptide CLKRSH-pS-DSLD coupled to keyhole limpet hemocyanin. Cells were lysed in mammalian cell lysis buffer (50 mM Tris·HCl,

Abbreviations: Chk, checkpoint kinase; Cdc, cell division cycle; IR, ionizing radiation; siRNA, small interfering RNAs; UV, UV light.

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pH 8/150 mM NaCl/0.5% Nonidet P-40/5 mM EDTA/2 mM DTT/1 mM sodium orthovanadate/10 mM β-glycerophosphate/1 mM NaF/1 μM microcystin/1 mM PMSF/5 μg/ml leupeptin/10 μg/ml aprotinin).

RNA Interference (RNAi) Experiments. RNAi was performed by using protocols supplied by Dharmacon Research (Lafayette, CO). The small interfering RNA (siRNA) oligonucleotide corresponding to nucleotides 127–147 of the human Chk1 coding region and nucleotides 82–102 of human Cdc25A were supplied by Dharmacon Research. siRNA duplexes were transfected into HeLa cells by using OLIGOFECTAMINE (Invitrogen) according to the manufacturer's recommendations. Unless indicated, cells were analyzed 36 h posttransfection. In some cases, cells were exposed to 6 or 10 Gy IR from a ^{60}Co source. Luciferase–siRNA was a gift from D. Dean (Washington University).

Monitoring the IR-Induced G_2 Checkpoint. HeLa cells were mock-transfected or transfected with duplex siRNAs. Thirty-six hours later, cells were mock-irradiated or exposed to 6 Gy IR from a ^{60}Co source. Cells were stained with 30 μg/ml of propidium iodide and for phospho-histone H3 1 h later. The procedure for immunofluorescent detection of phosphorylated histone H3 has been described (22). Cells were analyzed by flow cytometry by using a Becton–Dickinson FACScan. The data were analyzed by using CELLQUEST software. Chromosome spreading was performed as described (23). A minimum of 500 nuclei were counted for each sample and experiments were performed three times.

Histone H1 Kinase Assays. Cells were lysed in mammalian cell lysis buffer and incubated with cyclin B1-specific antibody (21). Precipitates were washed twice in incomplete kinase buffer (50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/1 mM DTT/10 mM β-glycerophosphate) and then incubated in 50 μl of complete kinase buffer (50 mM Tris, pH 7.4/10 mM $MgCl_2$/1 mM DTT/20 mM β-glycerophosphate/50 μm ATP/10 μCi [γ-^{32}P]) containing 1 μg of histone H1 for 10 min at 30°C. ^{32}P incorporation into histone H1 was quantified by using the STORM 860 imaging system (Amersham Pharmacia).

UCN-01 and Cycloheximide Treatment. Human diploid fibroblasts were incubated with 1 μM UCN-01 for 30 min and then either mock-irradiated or exposed to 6 Gy IR. Cells were harvested for Western blotting 1 h later. HeLa cells that had been transfected with Chk1-siRNA for 36 h were exposed to 6 Gy IR. Cycloheximide (25 μg/ml) was added, and cells were harvested at the indicated times.

Radio-Resistant DNA Synthesis. Inhibition of DNA synthesis after irradiation was assessed as described (24) except for the following modifications. Cells were incubated with siRNA in the presence of 10 nCi/ml [^{14}C]thymidine (NEN) for 24 h, then washed and incubated in DMEM for 12 h. Cells were either mock-irradiated or exposed to 5, 10, or 20 Gy IR, incubated for 1 h, and then pulse-labeled with 2.5 μCi/ml [^{3}H]thymidine (NEN) for 15 min. Cells were harvested, washed twice in PBS, and then incubated in 100% methanol for 5 min, followed by 10% trichloroacetic acid for 5 min, followed by 0.3 N NaOH. After neutralization with HCl, radioactivity was determined by liquid scintillation counting. The resulting ratios of ^{3}H cpm to ^{14}C cpm, corrected for cpm that resulted from channel crossover, were a measure of DNA synthesis.

Labeling and Mapping Studies. HeLa cells infected with recombinant adenovirus encoding myc-Cdc25A were incubated for 2 h in phosphate-free DMEM containing 2 mCi per ml of ^{32}P-labeled inorganic phosphate. Cdc25A was immunoprecipitated with anti-myc monoclonal antibody-agarose (Santa Cruz Biotechnology). GST-Chk1 was purified from baculoviral-infected Sf9 cells and 6xHis-Cdc25A and 6xHis-Cdc25A(S123A) were purified from *Escherichia coli*. Kinase reactions were carried out in 50 μl of complete kinase buffer for 10 min at 30°C. Proteins were resolved by SDS/PAGE, and radiolabeled Cdc25A was subjected to digestion with 2 μg/ml trypsin followed by 1 μg/ml chymotrypsin. Two-dimensional phosphopeptide mapping was performed as described (14).

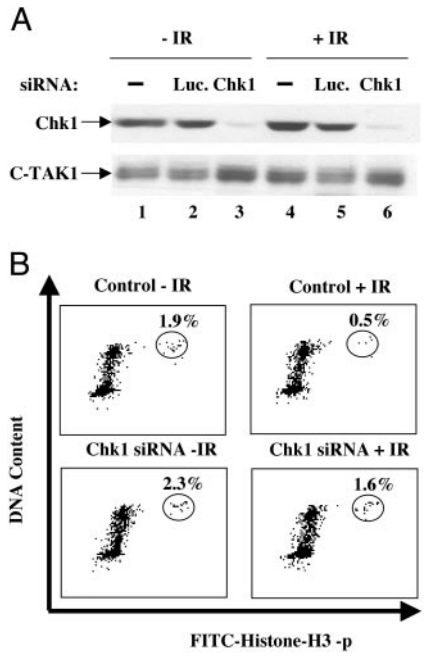

Fig. 1. Chk1 is required for the IR-induced G_2 checkpoint. Cells were mock-transfected (control) or transfected with double-strand siRNAs specific for either Chk1 or luciferase for 36 h. Cells were mock- or γ-irradiated, and 1 h later were harvested for Western blotting or were costained for DNA content and histone H3 phosphorylation and analyzed by flow cytometry (22). Percent mitotic cells is indicated.

BrdUrd Labeling. HeLa cells were mock-transfected or transfected with duplex siRNAs. Thirty-six hours later, cells were pulse-labeled for 20 min with 20 μM BrdUrd (Amersham Pharmacia). Cells were stained with 30 μg/ml propidium iodide and for BrdUrd with FITC-conjugated monoclonal antibody (Caltag, South San Francisco, CA) and processed for flow cytometric analysis at the times indicated.

Results

Chk1 Is Required for the IR-Induced G_2 Checkpoint. RNAi experiments were performed to determine whether Chk1 levels could be lowered in HeLa cells. As seen in Fig. 1*A*, Chk1 protein levels were significantly reduced in cells that had been incubated with Chk1-specific siRNAs (lanes 3 and 6). Chk1 levels were not reduced in cells treated with luciferase–siRNA (Fig. 1*A*, lanes 2 and 5), and C-TAK1 (20, 25) levels were not altered by RNAi treatment. Next, RNAi experiments were performed to determine whether Chk1 is required for the IR-induced G_2 checkpoint (Fig. 1*B*). The ability of G_2 cells in control and Chk1-siRNA-treated cultures were monitored for their ability to enter into mitosis within 1 h after IR. Cells were costained with propidium iodide to assess DNA content and with antiphosphohistone-H3 antibody to identify mitotic cells (22, 26). This experimental paradigm determines the percentage of G_2 cells that move into mitosis in the absence and

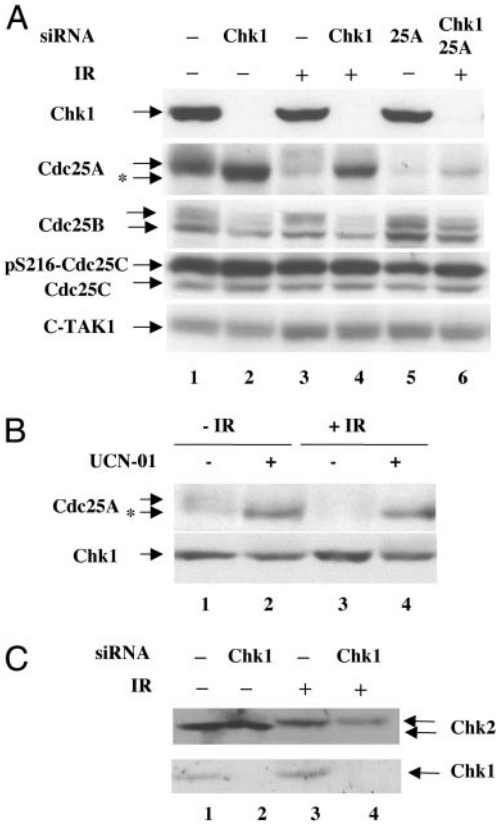

Fig. 2. Interactions between Chk1 and Cdc25A. (*A*) HeLa cells were mock-transfected or transfected with Chk1-siRNA, Cdc25A-siRNA, or both for 36 h. Cells were mock- or γ-irradiated and analyzed 1 h later by Western blotting. *, The fastest electrophoretic form of Cdc25A. The slower electrophoretic form of Cdc25C is phosphorylated on serine-216 (17, 20). (*B*) Normal human diploid fibroblasts were incubated with or without UCN-01 and then mock- or γ-irradiated. Cdc25A and Chk1 were visualized by Western blotting. (*C*) HeLa cells were mock-transfected or transfected with Chk1-siRNA for 36 h. Cells were then mock- or γ-irradiated and analyzed 1 h later for Chk2 by Western blotting.

presence of double-stranded DNA breaks (22, 26). Control (Fig. 1*B*) and luciferase–siRNA-treated cells (Fig. 8, which is published as supporting information on the PNAS web site) showed a 74% and 70% decrease, respectively, in the number of mitotic cells 1 h after IR. In contrast, Chk1-siRNA-treated cells showed only a 30% reduction in mitotic cells after IR. Chromosome spreading gave similar results to those obtained by phosphohistone–H3 staining, and histone H1 kinase assays revealed that Cdc2/cyclin B1 complexes were still active after exposure of Chk1-siRNA-treated cells to IR (see Figs. 9 and 10, which are published as supporting information on the PNAS web site). Cells expressing kinase-inactive Chk1 or a phosphorylation-site mutant (S315A, S345A) that fails to be activated in a checkpoint-dependent manner (14) were also compromised in their ability to delay in G_2 after IR (data not shown). Taken together these results demonstrate that Chk1 is required for the IR-induced G_2 checkpoint. Chk1-siRNA-treated cells experienced an S-phase delay, indicating an important S-phase function for Chk1 even in the absence of IR (data not shown).

Chk1 reportedly phosphorylates Cdc25A and Cdc25C (15, 17, 19). The fastest electrophoretic form of Cdc25A was observed to accumulate in Chk1-deficient cells suggesting a reduction in Cdc25A phosphorylation in these cells (denoted by an asterisk in Fig. 2*A*). Irradiation of control cells resulted in the loss of Cdc25A (Fig. 2*A*, compare lanes 1 and 3). In contrast, Cdc25A remained stable when Chk1-deficient cells were irradiated (Fig. 2*A*, compare lanes 2 and 4). Cycloheximide treatment confirmed that loss of Chk1 resulted in stabilization of Cdc25A protein after IR (see Fig. 11, which is published as supporting information on the PNAS web site). A reduction in the slower electrophoretic forms of Cdc25B was also observed in Chk1-siRNA-treated cells. Cdc25C phosphorylation status was not altered in Chk1-deficient cells and C-TAK1 was unaffected by loss of Chk1. RNAi technology was also used to determine whether Cdc25A levels could be reduced in Chk1-deficient cells. As seen in Fig. 2*A* (lane 6), we were able to reduce but not eliminate Cdc25A in Chk1-deficient cells. The partial reduction of Cdc25A protein observed in cells cotransfected with Chk1- and Cdc25A-siRNAs could be due to the longer half-life of Cdc25A in Chk1-deficient cells or to less efficient elimination of RNA in doubly transfected cells. However, Chk1 levels were reduced to similar levels in singly and doubly transfected cells

As an independent approach to determine whether Cdc25A is regulated by Chk1 after IR, we treated normal human diploid fibroblasts with the Chk1 inhibitor UCN-01 (10–12, 27). As seen in Fig. 2*B*, the fastest electrophoretic form of Cdc25A accumulated in cells where Chk1 activity was blocked by UCN-01 (lanes 2 and 4), which occurred in both the absence and presence of IR. Chk2 has been reported to phosphorylate Cdc25A after IR, thereby targeting Cdc25A for ubiquitin-mediated proteolysis (28). As seen in Fig. 2*C*, Chk2 levels were unaltered in Chk1-siRNA-treated cells, and Chk2 was fully phosphorylated when Chk1-siRNA-treated cells were exposed to IR (lane 4). Thus, in the absence of Chk1, Chk2 is unable to induce the degradation of Cdc25A in an IR-dependent manner.

Reducing Cdc25A Levels Partially Restores the G_2–DNA Damage Checkpoint in Chk1-Deficient Cells. Loss of Chk1 interferes with the ability of cells to eliminate Cdc25A after IR (Fig. 2), and loss of Chk1 causes bypass of the IR-induced G_2 checkpoint (Fig. 1). To determine whether elevated levels of Cdc25A contributed to checkpoint abrogation in Chk1-deficient cells, we tested whether the IR-induced G_2 checkpoint could be restored by reducing Cdc25A levels. Reduction in Cdc25A levels was observed in cells transfected with Cdc25A-siRNA (Fig. 3*A*, lane 3). As noted earlier, the fastest electrophoretic form of Cdc25A accumulated in cells transfected with Chk1-siRNA (Fig. 3*A*, lane 2), and this form was stable after irradiation (Fig. 3*A*, lane 6). We were able to reduce but not eliminate Cdc25A in irradiated Chk1-deficient cells by Cdc25A-siRNA cotransfection (Fig. 3*A*, lane 8). Cdc25C levels and phosphorylation status were not altered under any of the experimental conditions. Next, the ability of these cells to delay in G_2 after IR was examined (Fig. 3*B*). Fewer mitotic cells were observed in Cdc25A-siRNA-treated cells relative to control cells. Control cells and cells transfected with Cdc25A-siRNA showed an ≈80 and an ≈70% decrease, respectively, in the number of mitotic cells after IR, whereas cells transfected with Chk1-siRNA showed only a 22% decrease. Importantly, cells transfected with siRNAs for both Chk1 and Cdc25A partially regained their ability to delay in G_2 as a 50% decrease in the number of mitotic cells was observed after IR (Fig. 3*B*). Thus, lowering Cdc25A levels in Chk1-deficient cells is sufficient to partially restore G_2 cell cycle delay after IR. The incomplete restoration of G_2 delay in Cdc25A-siRNA-treated cells may be due to the remaining Cdc25A protein (Fig. 3*A*, lane 8) or to other pathways that are deregulated in the absence of Chk1.

Chk1 Is Required for the IR-Induced S-Phase Checkpoint. To determine whether Chk1 is also required for the IR-induced S-phase checkpoint, [^{3}H]thymidine incorporation into DNA was moni-

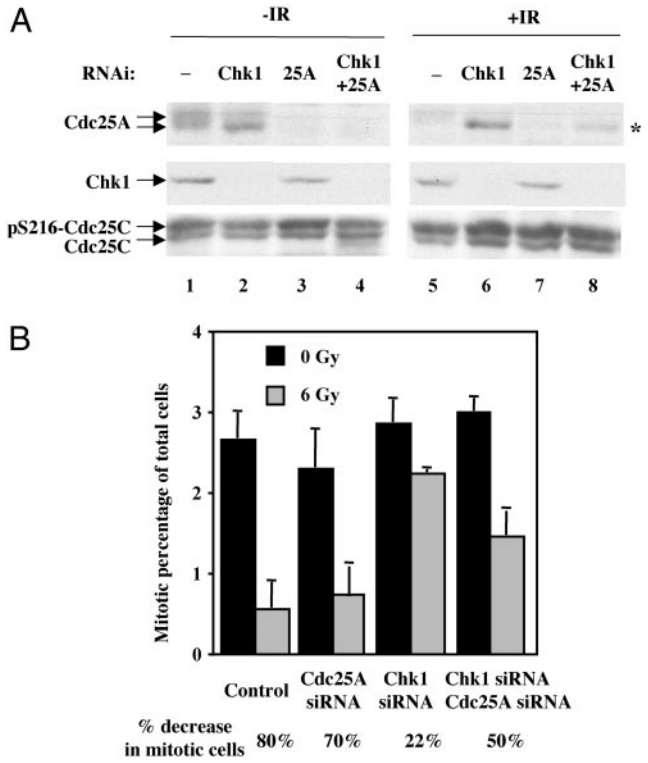

Fig. 3. Cdc25A is an effector of Chk1. (*A*) HeLa cells were mock-transfected or transfected with duplex siRNAs specific for Chk1, Cdc25A, or both. Cells were lysed for Western blotting. The slower electrophoretic form of Cdc25C is phosphorylated on serine-216. *, The fastest electrophoretic form of Cdc25A. (*B*) HeLa cells were mock-transfected (control) or transfected with duplex siRNAs specific for Chk1, Cdc25A, or both. Cells were lysed for Western blotting (*A*) or stained for both DNA content and histone H3 phosphorylation. Cells were analyzed by flow cytometry 1 h after mock or γ irradiation. Mitotic cells are represented as a percentage of total cells before and after IR. The error bars represent the standard deviation after combining results from at least three experiments for each condition. Percent decrease in mitotic cells after IR is indicated.

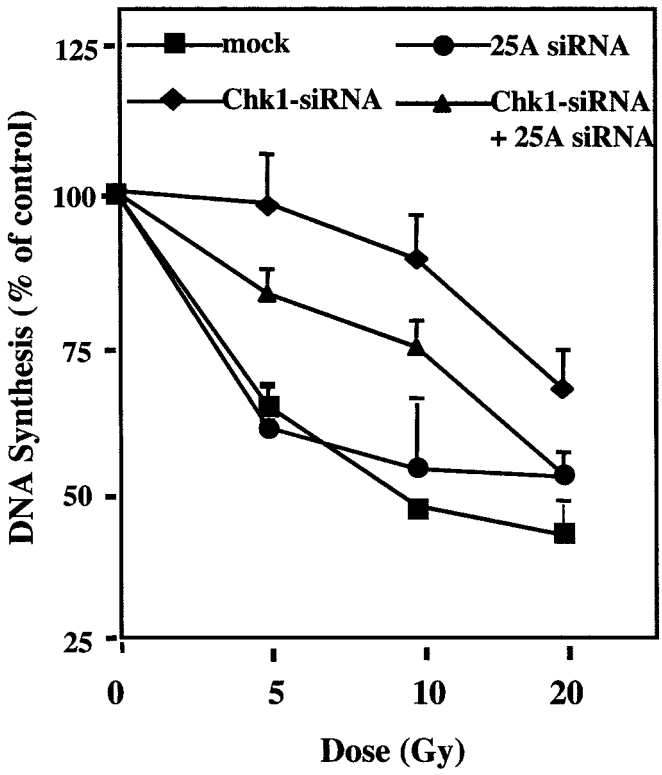

Fig. 4. Chk1 is required for the IR-induced S-phase checkpoint. Radioresistant DNA synthesis was assessed 1 h after various doses of IR in control and siRNA-treated cells.

tored 1 h after exposure of cells to various doses of IR (Fig. 4). Control cells had an intact checkpoint as indicated by the dose-dependent decrease in [^{3}H]thymidine incorporation into newly synthesized DNA. In contrast, cells treated with Chk1-siRNA continued to initiate DNA replication as indicated by the radio-resistant DNA synthesis at all doses of IR. Furthermore, cyclin E-associated kinase activity was not reduced in Chk1-siRNA-treated samples after IR (data not shown). Importantly, treatment of Chk1-deficient cells with Cdc25A-siRNA partially rescued the IR-induced S-phase checkpoint.

Chk1 Regulates Cdc25A Phosphorylation. The fastest electrophoretic form of Cdc25A accumulates in Chk1-deficient cells (Figs. 2*A* and 3*A*). Labeling experiments were carried out *in vivo* to monitor the phosphorylation status of this form of Cdc25A. As seen in Fig. 5*A*, multiple-phosphorylated forms of Cdc25A that run as a smear on SDS gels were observed in control cells (lane 1). In contrast, a faster electrophoretic form of Cdc25A was seen in Chk1-deficient cells, and this form was poorly phosphorylated *in vivo* (Fig. 5*A*, lane 2). These results suggest that Chk1 either directly or indirectly regulates the phosphorylation of Cdc25A *in vivo*. Phosphorylation of Cdc25A on S123 facilitates the proteasome-dependent degradation of Cdc25A (19, 28). We generated an antibody specific for Cdc25A when it is phosphorylated on serine-123 (Fig. 5*B*) and used this antibody to determine whether Cdc25A is phosphorylated on S123 in the absence of DNA damage (Fig. 5*C*). Lysates prepared from asynchronously growing HeLa cells were resolved directly by SDS/PAGE (Fig. 5*C*, lane 1) or were first precipitated with protein A beads alone (Fig. 5*C*, lane 2) or with antibody specific for Cdc25A (Fig. 5*C*, lane 3). Western blotting was performed with antibodies specific for Cdc25A (Fig. 5*C*, *Upper*) or with the S123 phospho-specific antibody (Fig. 5*C*, *Lower*). As seen in Fig. 5*C*, Cdc25A was phosphorylated on S123 in the absence of checkpoint activation and this likely contributes its instability during an unperturbed cell cycle. Next, labeling experiments were carried out *in vitro* and *in vivo* to determine whether Cdc25A is a substrate of Chk1 and whether Chk1 directly phosphorylates Cdc25A on serine-123. Chk1 phosphorylated Cdc25A on S123 *in vitro* (Fig. 5, *D* and *E*) and Cdc25A was phosphorylated on S123 *in vivo* (Fig. 5*F*). The phosphorylation pattern of Cdc25A labeled *in vivo* (Fig. 5*F*) was remarkably similar to that of Cdc25A phosphorylated by Chk1 *in vitro* (Fig. 5*D*), suggesting that Chk1 is a key Cdc25A-kinase *in vivo*.

Discussion

In this study, we have investigated the contribution made by the Chk1 protein kinase to the regulation of the cell division cycle. We report that a hypophosphorylated form of Cdc25A accumulates in Chk1-deficient cells, and that Chk1 phosphorylates Cdc25A *in vitro* on identical sites phosphorylated *in vivo*. Taken together, these results implicate Chk1 as a direct regulator of the Cdc25A protein phosphatase *in vivo*. *Chk1* is an essential gene in mice (8, 9), and one of its essential noncheckpoint functions may be to negatively regulate Cdc25A to avoid inappropriate cell cycle advancement throughout early embryonic development.

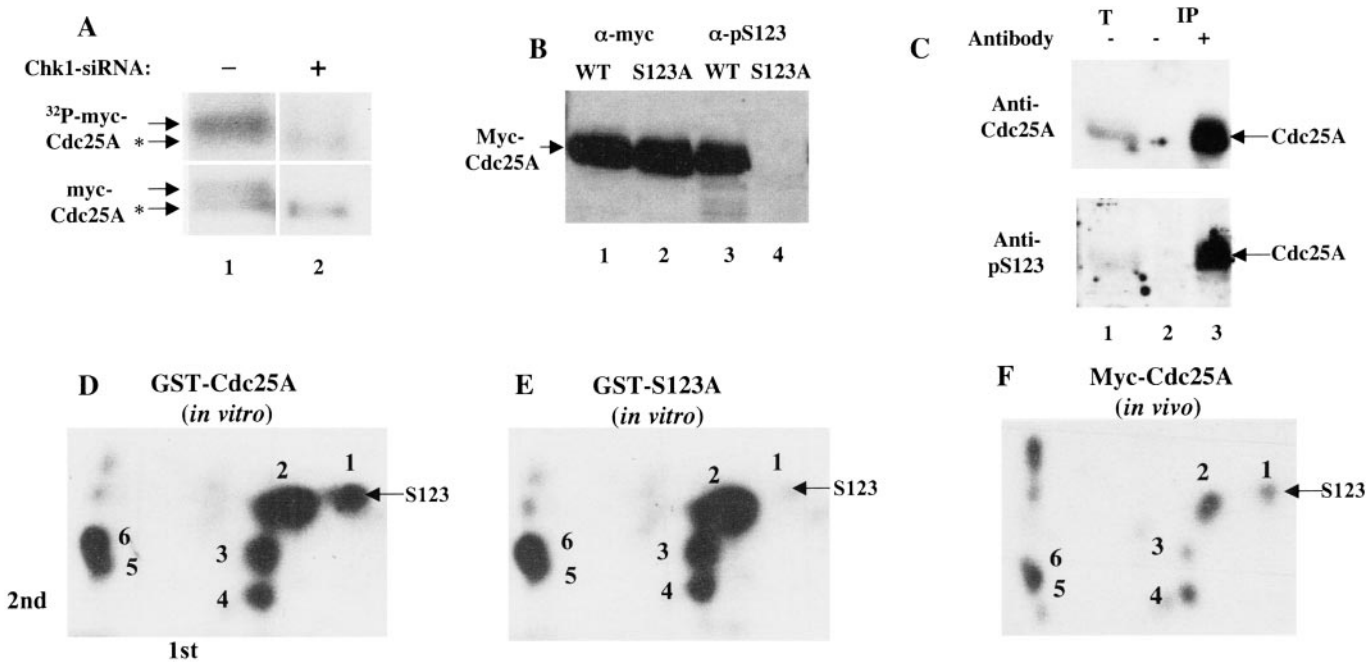

Fig. 5. Dependence of Cdc25A phosphorylation on Chk1. (*A*) HeLa cells that had been mock-transfected or transfected with Chk1-siRNA for 36 h were infected with recombinant adenovirus encoding myc-tagged human Cdc25A for 4 h. Cells were incubated with ^{32}P-labeled inorganic phosphate, and after 2 h Cdc25A was immunoprecipitated and resolved by SDS/PAGE and then blotted onto nitrocellulose. Radiolabeled Cdc25A was visualized by autoradiography (*Upper*) and then subjected to Western blotting (*Lower*). *, The fastest electrophoretic form of Cdc25A. (*B*) Lysates from HeLa cells transfected with plasmids encoding myc-Cdc25A (WT) and myc-Cdc25 (S123A) were incubated with anti-c-Myc mouse monoclonal 9E10-conjugated agarose. Precipitates were resolved by SDS/PAGE and immunoblotted with antibodies specific for the myc-tag (lanes 1 and 2) or the pS123-specific antibody (lanes 3 and 4). (*C*) HeLa cell lysates were resolved directly by SDS/PAGE (T, lane 1) or were first precipitated in the absence (lane 2) or presence (lane 3) of Cdc25A-specific antibody. Proteins were detected by immunoblotting with antibodies specific for Cdc25A (*Upper*) or with the pS123 antibody (*Lower*). (*D–F*) Radiolabeled Cdc25A (*D*) and Cdc25A(S123A) (*E*) phosphorylated by Chk1 *in vitro* and myc-tagged Cdc25A (*F*) labeled *in vivo* were subjected to 2D phosphopeptide mapping. Arrows indicate the direction of the first and second dimensions.

Ionizing radiation induces the degradation of Cdc25A, clearly a Chk1-dependent process, because in the absence of Chk1, Cdc25A levels remained high. Loss of Chk1 and elevated levels of Cdc25A correlated with bypass of the IR-induced S and G_2 checkpoints. Both checkpoints could be partially restored by interfering with Cdc25A accumulation in Chk1-deficient cells. These findings argue that negative regulation of Cdc25A by Chk1 is essential for cells to delay cell cycle progression in the presence of double-strand DNA breaks. Previous studies reported that the negative regulation of Cdc25A by Chk1 and Chk2 occurred in a checkpoint-dependent manner after exposure of cells to genotoxic stress (19, 28). Importantly, this study demonstrates that Chk1 regulates Cdc25A in the absence of checkpoint activation. Furthermore, IR-activated Chk2 failed to destabilize Cdc25A when Chk1 was eliminated by siRNA treatment (Fig. 2*C*). These data suggest that Chk1 continually phosphorylates Cdc25A during an unperturbed cell cycle on several sites, including S123, and that phosphorylation of Cdc25A by Chk1 is required for ionizing radiation to induce the degradation of Cdc25A (Fig. 6). Thus, Chk1 may "mark" Cdc25A so that it can be acted on by effectors of the DNA damage response only in its phosphorylated state. Future studies will be required to distinguish individual contributions made by Chk1 and Chk2 to Cdc25A regulation.

Several studies have reported that Chk1 regulates Cdc25C by phosphorylating serine-216 (15, 17, 29). Significant differences in the levels of serine-216 phosphorylation were not observed in Chk1-deficient cells either in the absence or in presence of IR (Figs. 2*A* and 3*A*). Thus, either Chk1 has no role in regulating serine-216 phosphorylation or loss of Chk1 is compensated by other cellular kinases. The protein kinase inhibitor UCN-01 is a potent inhibitor of Chk1, and Cdc25C becomes rapidly dephosphorylated on serine-216 in UCN-01-treated cells (10–12, 27). The findings reported here indicate that UCN-01 must target

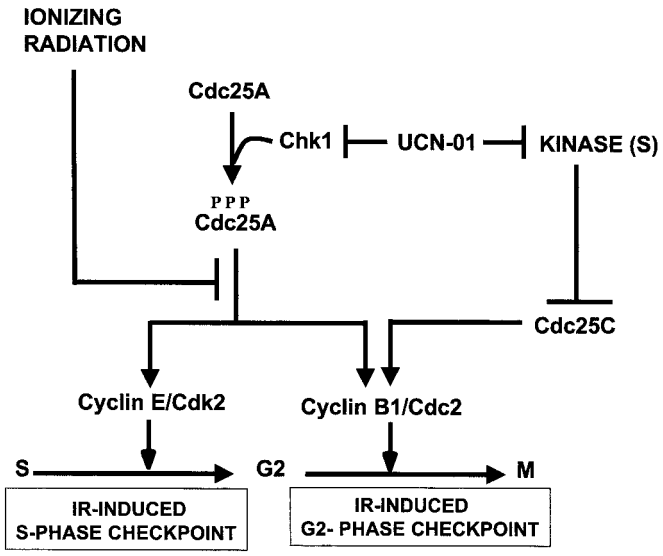

Fig. 6. Regulation of Cdc25A by Chk1. Throughout the cell division cycle, Cdc25A is continually being phosphorylated by Chk1 to promote its turnover. Ionizing radiation activates a pathway that targets phosphorylated Cdc25A for complete destruction. The anticancer agent UCN-01 abrogates G_2 checkpoint function by inhibiting Chk1 to deregulate the Cdc25A pathway and by inhibiting kinases other than Chk1 to deregulate the Cdc25C pathway (11).

kinases in addition to Chk1 to completely deregulate Cdc25C *in vivo*.

Cdc25A is thought to regulate the G_1- to S-phase transition (30–33), but several lines of evidence indicate that Cdc25A may also have a role in regulating the G_2- to M-phase transition. Cdc25A is present and active in all stages of the cell cycle, and Cdc25A levels actually rise as cells progress from S phase to mitosis (30–35). Cdc25A is capable of binding to cyclin B1 and can activate Cdc2/cyclin B1 complexes *in vitro* (36, 37), and neutralizing antibodies to Cdc25A arrests cells in G_2 (37). Overexpression of Cdc25A accelerates entry into mitosis, and expression of a phosphatase-inactive mutant of Cdc25A delays entry into mitosis (data not shown). Taken together, these findings support a role for Cdc25A in regulating mitotic entry during a normal cell cycle and legitimize it as a key target of Chk1 action in the S and G_2 phases of the cell cycle.

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