Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A

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Summary

Chk1 kinase coordinates cell cycle progression and preserves genome integrity. Here, we show that chemical or genetic ablation of human Chk1 triggered supraphysiological accumulation of the S phase-promoting Cdc25A phosphatase, prevented ionizing radiation (IR)-induced degradation of Cdc25A, and caused radioresistant DNA synthesis (RDS). The basal turnover of Cdc25A operating in unperturbed S phase required Chk1-dependent phosphorylation of serines 123, 178, 278, and 292. IR-induced acceleration of Cdc25A proteolysis correlated with increased phosphate incorporation into these residues generated by a combined action of Chk1 and Chk2 kinases. Finally, phosphorylation of Chk1 by ATM was required to fully accelerate the IR-induced degradation of Cdc25A. Our results provide evidence that the mammalian S phase checkpoint functions via amplification of physiologically operating, Chk1-dependent mechanisms.

Introduction

A growing number of genetic syndromes highly prone to cancer share an aberrant cellular response to DNA double-strand breaks (DSB), the most lethal type of DNA damage. When exposed to ionizing radiation (IR) or radiomimetic drugs, these cells are unable to slow down the rate of DNA replication and undergo the so-called radioresistant DNA synthesis (RDS) (Kastan and Lim, 2000; Rotman and Shiloh, 1999). RDS was first reported for cells derived from ataxia-telangiectasia (A-T) patients (Painter and Young, 1980), and later found in Nijmegen-breakage syndrome (NBS) (Khanna and Jackson, 2001), ataxia-telangiectasia-like disorder (A-TLD) (Stewart et al., 1999) and Fanconi anemia (FA) (Taniguchi et al., 2002). Moreover, cells derived from tumors with mutated BRCA1 (Xu et al., 2001) and Chk2 (Falck et al., 2001) tumor suppressor, respectively, also undergo RDS when irradiated. It has been proposed that in combination with defects in other cell cycle checkpoints, RDS may contribute to destabilize the genome and thereby predispose individuals suffering the above-mentioned genetic diseases to cancer (Rotman and Shiloh, 1999; Hartwell and Kastan, 1994).

The S phase checkpoint is an evolutionarily conserved signal transduction pathway responding to IR-induced DSBs by a rapid inhibition of origins of DNA replication (Kastan and Lim, 2000). The master (and the most proximal) transducer of the S phase checkpoint is ataxia-telangiectasia-mutated protein (ATM), a member of an evolutionarily conserved family of phosphatidyl inositol 3-like kinases (Rotman and Shiloh, 1999). One critical event downstream of ATM is degradation of the S phase-promoting Cdc25A phosphatase (Falck et al., 2001). IR-induced accelerated proteolysis of Cdc25A leads to inhibition of the Cdk2 kinase activity, which in turn prevents loading of the Cdc45 origin binding factor on chromatin (Costanzo et al., 2000; Falck et al., 2002). Lack of Cdc45 precludes recruitment of DNA polymerases, thereby blocking initiation of DNA replication from the unfired origins (Takisawa et al., 2000).

ATM propagates (and presumably amplifies) the signal through downstream effector kinases. One such mediator is Chk2, a Ser/Thr protein kinase, which requires phosphorylation

SIGNIFICANCE

Cancer cells commonly possess aberrations in both the cell cycle machinery and the checkpoint pathways activated by DNA damage. The Cdc25A phosphatase is a cell cycle regulatory protooncogene that is overexpressed in aggressive tumors, and a target of checkpoint pathways mediated by Chk1 and Chk2 kinases, two tumor suppressors mutated in diverse human malignancies. Here we report that Chk1 phosphorylates Cdc25A and thereby controls the Cdc25A protein turnover in unperturbed cell cycles. This mechanism is enhanced jointly by ATM-controlled Chk1 and Chk2 to provide a proper cell cycle-delay response to ionizing radiation. Our concept helps to elucidate the contributions of Cdc25A, Chk1, and Chk2 deregulation to oncogenesis, and suggests a way to sensitize cancer cells to radiotherapy.

by ATM for its activation (Matsuoka et al., 1998). Chk2 directly interacts with and phosphorylates Cdc25A in an ATM- and IR-dependent manner (Falck et al., 2001). However, the complexity of Cdc25A phosphorylation and the spectrum of potential kinases involved in this process are not completely understood. Primary embryonic fibroblasts (MEFs) from mice with homozygous disruption of the *Chk2* gene did not undergo RDS, although immortalized cell lines derived from such Chk2-deficient MEFs did show a delayed initiation and shorter duration of the S phase checkpoint (Hirao et al., 2002). These findings indicate that the absence of Chk2 during embryonic development could trigger some compensatory signaling pathway.

Accelerated proteolysis of Cdc25A was originally discovered as a cellular response to ultraviolet light (UV)-induced DNA damage (Mailand et al., 2000) and hydroxyurea (HU)-triggered stalling of replication forks (Molinari et al., 2000). Degradation of Cdc25A was also observed under physiological conditions during midblastula transition in *Xenopus* embryos (Shimuta et al., 2002). Phosphorylation (and the consequent destruction) of Cdc25A in all those cases is mediated by a Chk1 Ser/Thr protein kinase. Chk1 is structurally unrelated to Chk2, and despite other indications of their nonoverlapping functions (Hirao et al., 2002; Liu et al., 2000), there is evidence to suggest that at least in some responses to genotoxic stress, Chk1- and Chk2-controlled pathways might converge on shared targets. Most notably, Chk1 and Chk2 can phosphorylate the same substrates in vitro, including checkpoint regulators such as p53 (O'Neill et al., 2002; Shieh et al., 2000), Cdc25C (Matsuoka et al., 1998), and Cdc25A (Falck et al., 2001; Sanchez et al., 1997).

It has been speculated that Chk1 might be the factor that compensates for the lack of Chk2 in the S phase checkpoint in primary mouse cells (Hirao et al., 2002). Although plausible, a role of Chk1 in the rapid IR-induced intra-S-phase responses has not been directly demonstrated, and, in general, the specific function(s) of mammalian Chk1 in DSB-activated checkpoints is unclear. Rather than ATM, the major upstream activator of Chk1 appears to be ATR (ATM- and Rad3-related), another member of the PI3 kinase family, associated mainly with non-DSB types of DNA damage (Abraham, 2001). Apart from the uncertainty about the unique versus redundant roles of Chk1 and Chk2 in regulating Cdc25A degradation in response to distinct types of genotoxic stress, the observation that Cdc25A protein is unstable also in undamaged interphase cells (Mailand et al., 2000; Molinari et al., 2000) raises yet another conceptually important question. It is not known whether the damage-induced enhanced degradation of Cdc25A reflects an amplification of a mechanism that operates during each physiological S phase or whether it requires activation of a specialized pathway(s) that are dormant in unstressed cells.

In an attempt to address these questions, we performed a detailed analysis of Cdc25A phosphorylation in vitro and in vivo. We show that Chk1 controls the basal physiological turnover of Cdc25A through phosphorylation of four serine residues. After IR, the activated Chk2 coregulates a subset of these residues on Cdc25A. Furthermore, the IR-induced acute acceleration of Cdc25A proteolysis requires ongoing Chk1 activity, as well as ATM-mediated phosphorylations of Chk1. The switch between the basal and the accelerated proteolysis of Cdc25A is determined by the rate of phosphate turnover on the phosphoacceptor site(s) jointly targeted by Chk1 and Chk2, rather than any de novo checkpoint-specific phosphorylations. Our results provide mechanistic insights into how Chk1 and Chk2 cooperate to avoid potentially cancer-promoting consequences of unscheduled DNA replication in response to IR.

Results

Chk1 is required to control basal turnover and IR-induced downregulation of Cdc25A

To elucidate the role of Chk1 in the regulation of Cdc25A, we inhibited Chk1 in several different ways and assayed the impact on Cdc25A abundance and activity. First, addition of the Chk1 inhibitor UCN-01 (Busby et al., 2000; Graves et al., 2000) for 1 hr to human U2-OS cells in the absence of IR caused increased Cdc25A protein level and activity (Figure 1A, lane 2). In cells grown without any drug, the Cdc25A protein and activity rapidly declined after 10 Gy IR (Figure 1A, lane 3), yet this response was abrogated when UCN-01 was added immediately before (Figure 1A, lane 4) or after (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/3/3/247/DC1) irradiation. Consistent with the established role of Cdc25A in Cdk2 activation, cyclin E/Cdk2 and cyclin A/Cdk2 activities increased after UCN-01 treatment, similarly to the Cdc25A activity (Figure 1A). Next, we inhibited Chk1 by a new chemical inhibitor, CEP-3891, which is distinct from UCN-01 and likely more specific for Chk1 as, for example, it does not inhibit other UCN-01 targets such as c-Tak1 kinase (Supplemental Figure S2 and Supplemental Tables S1 and S2). Analogous to UCN-01, treatment of nonirradiated U2-OS cells with CEP-3891 caused a detectable increase in Cdc25A protein level and activity (Figure 1B, lane 3), and addition of CEP-3891 immediately before IR (Figure 1B, lane 4) diminished the rapid IR-induced downregulation of Cdc25A.

To validate the experiments performed by chemical inhibitors, we downregulated Chk1 expression by RNA interference (RNAi). HeLa cells transfected with Chk1-directed short inhibitory RNA (siRNA) oligonucleotides grossly reduced the Chk1 protein abundance and kinase activity (Figure 1C). Consistent with the effects seen with both chemical inhibitors of Chk1, Cdc25A protein level and activity increased in siRNA-transfected, nonirradiated cells (Figure 1C, lane 5), and the IR-induced downregulation of Cdc25A was impaired (Figure 1C, lane 6). Thus, three independent tools of Chk1 inhibition lead to Cdc25A stabilization in unstressed cells and prevented its efficient degradation after IR. The specificity of Chk1 inhibition in our assays was confirmed by the lack of any impact of the Chk1-targeting tools on Chk2 kinase activity toward Cdc25A (Figure 1D). Collectively, these data suggest that Chk1 controls both the basal and the IR-induced accelerated turnover of Cdc25A.

Identification and characterization of the Chk1-targeted phosphorylation sites of Cdc25A in vivo

The above results suggested that Chk1 regulates Cdc25A via direct phosphorylation. To explore Cdc25A phosphorylation in vivo, U2-OS cells were transfected with a tetracycline inducible HA-tagged Cdc25A, an approach used to avoid any deleterious effects of prolonged overexpression of Cdc25A. Cdc25A was induced for 6 hr, radiolabeled with 32P-ortho-phosphate, immunopurified, and used to generate phosphopeptide maps (Figure 2A). Over 20 phosphopeptides were detectable, and since similar patterns were obtained using the much weaker isotope 33P-ortho-phosphate (data not shown), these results do not seem to be biased by 32P-induced damage during the labeling period.

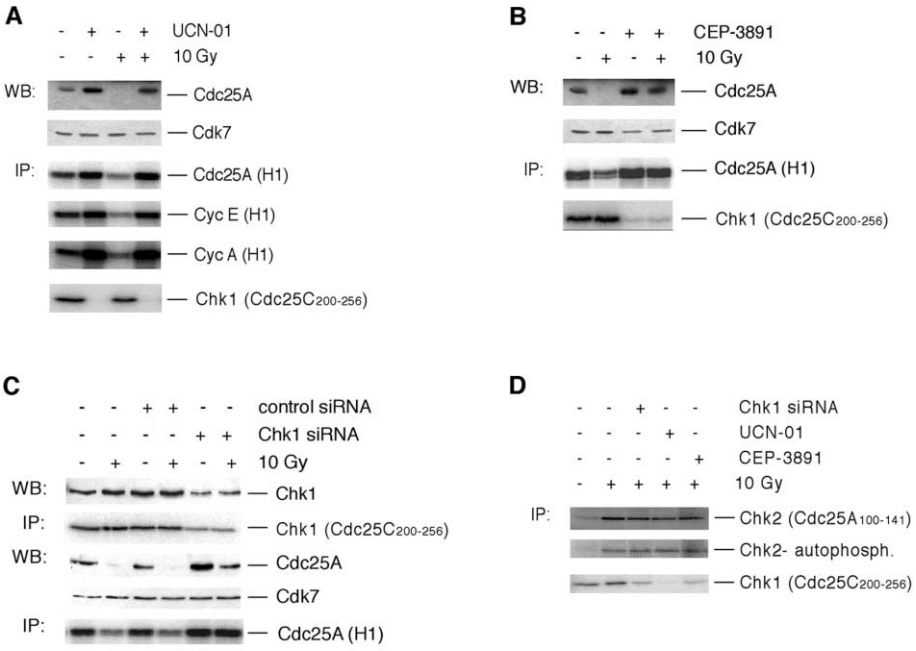

Figure 1. Chk1 controls Cdc25A protein level in both unstressed and irradiated cells

A: Chk1 inhibition by UCN-01 causes increased amount of Cdc25A protein and phosphatase activity, and increased cyclin E/A kinase activities. Extracts were prepared from U2-OS cells at 1 hr after IR. UCN-01 was added immediately prior to irradiation. Inhibition of Chk1 was confirmed by an in vitro kinase assay with or without UCN-01. Cdk7 protein levels demonstrate equal protein input.

B: Chk1 inhibition by CEP-3891 enhances the Cdc25A protein level and phosphatase activity. U2-OS cells were treated with CEP-3891, irradiated, and 1 hr later analyzed by Western blotting. Inhibition of Chk1 was confirmed by an in vitro kinase assay with or without CEP-3891.

C: Chk1 inhibition by siRNA causes increased amount of Cdc25A protein and phosphatase activity. HeLa cells were irradiated at 34 hr after transfection with control or Chk1 siRNA and extracts were prepared 1 hr later. Reduced expression of Chk1 protein was confirmed by Western blotting and in vitro kinase assay.

D: Chk2 kinase is not inhibited by Chk1 inhibition. In vitro kinase assays for Chk1 and Chk2 are shown in the absence and presence of Chk1 inhibitors. Chk2 autophosphorylation is also shown. Extracts from HeLa cells were prepared at 1 hr after irradiation of cells pretreated with Chk1 inhibitors for 15 min (UCN-01 and CEP-3891) or 34 hr (siRNA-transfection).

When 33P-ortho-phosphate was used to label the cells, IR exposure (15 Gy) did not lead to any major change of the phosphopeptide pattern, suggesting that IR does not induce de novo phosphorylations on Cdc25A (negative data not shown). Importantly, inhibition of Chk1 by addition of UCN-01 (Figure 2B) or CEP3891 (Supplemental Figure S3 at http://www.cancercell.org/cgi/content/full/3/3/247/DC1) led to a clear reduction in phosphorylation of at least 4 residues on Cdc25A. This shows that Chk1 phosphorylates Cdc25A in vivo on residues that might be critical for its stability.

The precise identity of the individual phosphopeptides was determined by phosphoamino acid analysis and cycle sequencing of the tryptic phosphopeptides from in vivo labeled Cdc25A, combined with site-directed mutagenesis. We identified the four Chk1-targeted phosphorylated sites as serines 123, 178, 278, and 292 (Figure 2C and Supplemental Figure S4). The spot containing S178 was not completely eliminated upon alanine substitution of S178 due to comigration of two phosphopeptides (data not shown). To rule out overexpression-associated adverse effects, phosphopeptide maps of endogenous Cdc25A were determined, and the Chk1-regulated sites confirmed (Figure 2D). Together, these data suggested that Chk1 phosphorylates Cdc25A on four residues.

Chk2 phosphorylates a subset of the Chk1-targeted sites of Cdc25A

To confirm that the in vivo-identified sites of Cdc25A could be directly phosphorylated by Chk1, a GST-Cdc25A fusion protein was phosphorylated in vitro with recombinant Chk1. Among the sites, which were phosphorylated, we could identify S123, S178, S278, and S292 (Figure 3A). Although phosphorylated S123 often appeared as a smear, rather than a spot, its identity was confirmed by phosphopeptide mapping of GST-Cdc25A where S123 was altered to alanine (data not shown).

We next phosphorylated GST-Cdc25A in vitro with recombinant Chk2 (Figure 3B). Chk2 phosphorylated Cdc25A in a manner partly overlapping with Chk1. However, the site identified as S278 was almost undetectable upon phosphorylation with Chk2, while the spots corresponding to phosphorylation on S123, S178, and S292 were clearly detectable. The similarity of the Chk1 and Chk2 phosphorylation patterns was confirmed by creating a map where we combined GST-Cdc25A that had been separately phosphorylated by Chk1 and Chk2. Indeed, the phosphopeptides corresponding to S123, S178, and S292 clearly comigrated (Figure 3C). Compared to these 3 phosphopeptides, the S278 spot was weaker in the combined map than in the Chk1-exclusive map, indicating that this peptide is almost exclusively phosphorylated by Chk1 (Figure 3C). The fact that Chk2 could only phosphorylate a subset of the sites suggests that the roles of Chk1 and Chk2 in regulation of Cdc25A may not be simply redundant.

Phosphorylation of serines 123, 178, 278, and 292 regulates both basal and IR-induced accelerated proteolysis of Cdc25A

We next tested phosphorylation-site mutants of Cdc25A for their ability to be downregulated in the absence or presence of IR. Consistent with our previous results (Falck et al., 2001), the turnover of wild-type Cdc25A was accelerated in cells exposed to IR (Figure 4A, upper panel). We then generated a Cdc25A mutant (S123A, S178A, S278A, and S292A) that lacks all phospho-sites identified in this study. This quadruple mutant was significantly more stable than the wild-type Cdc25A regardless

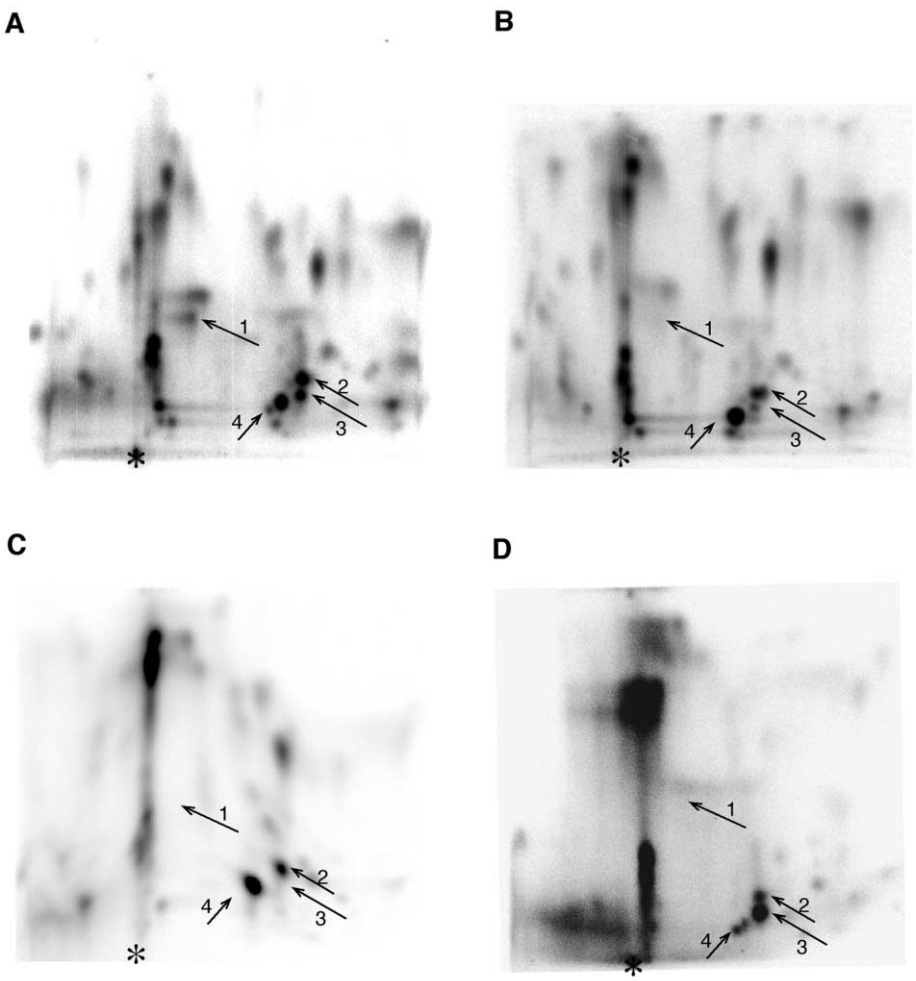

Figure 2. Chk1 phosphorylates Cdc25A on S123, S178, S278, and S292 in vivo

A: Phosphopeptide map of in vivo [^{32}P]-labeled HA-tagged Cdc25A from U2-OS cells. Expression of HA-Cdc25A was induced by tetracycline removal for 6 hr prior to the labeling with [^{32}P]-orthophosphate (0.5 mCi/ml) for 2 hr. The immunopurified HA-Cdc25A was processed for phosphopeptide mapping.

B: Addition of UCN-01 reduces the phosphorylation of Cdc25A on specific residues in vivo. U2-OS cells were treated with UCN-01 during the [^{32}P]-labeling period and analyzed as in **A**.

C: S123, S178, S278, and S292 are major UCN-01-sensitive phosphorylation sites of Cdc25A. Phosphopeptide mapping was performed as in **A** with an inducible HA-Cdc25A construct harboring alanine substitutions of S123, S178, S278, and S292.

D: Phosphopeptide mapping of endogenous Cdc25A from U2-OS cells.

Parental U2-OS cells were labeled and subjected to phosphopeptide mapping. Asterisks indicate the sample application points. Arrows and numbers indicate the following phosphopeptides: (1) S123, (2) S178, (3) S292, and (4) S278.

of IR exposure in both cycloheximide (Figure 4A, lower panel) and 35S-methionine-based (Figure 4B) pulse-chase assays.

We and others previously showed that mutation of serine 123 alone was sufficient to impair the IR-induced Cdc25A degradation (Falck et al., 2001; Donzelli et al., 2002). We therefore compared the stability of a Cdc25A-S123A mutant with the quadruple mutant or various combinations of mutations (Figure 4C and data not shown). These experiments confirmed that S123 is indeed a prominent residue involved in regulating the Cdc25A protein turnover, as this was the only site that affected the Cdc25A half-life when mutated alone, and prevented the IR-induced degradation (Figure 4C and data not shown). Adding two more mutations to S123 in a triple mutant had no additional effect. However, the quadruple mutant was more stable than Cdc25A-S123A both with and without irradiation (Figure 4C). Thus, concomitant phosphorylation of the four serines 123, 178, 278, and 292 is required for optimal regulation of Cdc25A stability.

The rate of phosphate exchange on the Chk1/Chk2-targeted S123 is modulated by IR

To study how Chk1- and Chk2-mediated phosphorylations regulate the turnover of Cdc25A, we used an antibody against phosphoserine123 (anti-pS123). This residue was chosen because in our assays, it was the only single phosphorylation site whose mutation clearly affected the stability of Cdc25A. The antibody recognized Cdc25A prephosphorylated in vitro with either Chk1 or Chk2 (Figure 5A), and its specificity was documented by the lack of reactivity with similarly prephosphorylated Cdc25A-S123A. Western blotting with anti-pS123 on lysates from IR-exposed versus untreated cells showed that S123 was already phosphorylated in undamaged cells (data not shown). This result is consistent with our phosphopeptide mapping data, which did not identify any de novo DNA damage-induced phospho-sites on Cdc25A.

We then assayed the kinetics of Cdc25A phosphorylation in cell extracts, using the pS123 antibody. In extracts from undamaged cells, S123 was phosphorylated after 5 min of incubation, and after 20 min, Cdc25A appeared in shifted forms (Figure 5B). In contrast, after IR exposure, the kinetics of S123 phosphorylation were faster and Cdc25A shifted more rapidly to slower migrating forms. Importantly, the chemical inhibition of Chk1 or Chk2 resulted in a delay in the IR-induced S123 phosphorylation, and this effect was consistently augmented when both inhibitors were combined (Figure 5C). These data indicate that after IR-induced DNA damage, both Chk1 and Chk2 participate in phosphorylating Cdc25A on a residue critical for its stability. To validate the latter conclusion in vivo, we

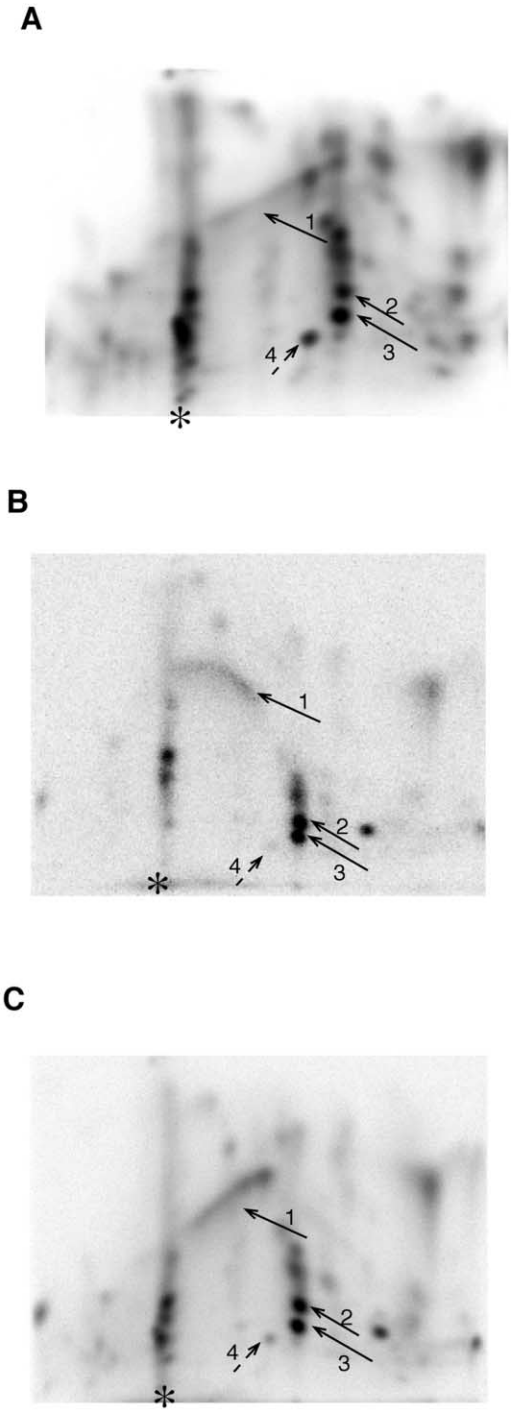

Figure 3. Chk1 and Chk2 share partly overlapping phosphorylation sites on Cdc25A in vitro

A: Phosphopeptide map of GST-Cdc25A phosphorylated in vitro with recombinant Chk1 shows phosphorylation of S123, S178, S278, and S292.
B: Recombinant Chk2 phosphorylates S123, S178, and S292 on GST-Cdc25A in vitro.
C: Comigration of S123, S178, and S292 phosphopeptides after mixing equal amounts of GST-Cdc25A separately phosphorylated by Chk1 and Chk2, respectively. Asterisks indicate the sample application points. Plain arrows point to the S123, S178, and S292 phosphopeptides shared by Chk1 and Chk2; dashed arrow indicates S278, specific for Chk1.

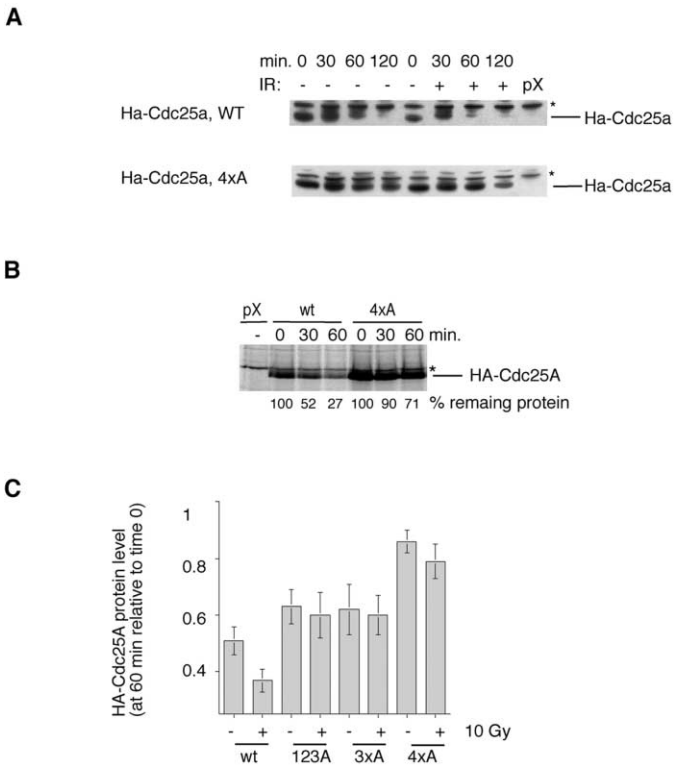

Figure 4. Cdc25A protein stability is dependent on phosphorylation of S123, S178, S278 and S292 regardless of DNA damage

A: Increased stability of the Cdc25A-4xA mutant in U2-OS cells. At sixteen hr after transfection with wild-type HA-Cdc25A or the Cdc25A-4xA mutant, cells were irradiated with 10 Gy as indicated, and cycloheximide was added immediately to block new protein synthesis. At the indicated time points, whole-cell lysates were immunoblotted with the anti-HA antibody. Asterisks indicate a crossreacting protein shown here as a loading marker, and pX indicates cells transfected with empty vector.
B: Increased stability of the Cdc25A-4xA mutant in a [^{35}S]-methionine pulse-chase. U2-OS cells were transfected with wild-type HA-Cdc25A or HA-Cdc25A-4xA expression plasmids. Sixteen hr later, the cells were pulsed with [^{35}S]-methionine (0.1 mCi/ml) for 30 min and harvested at the indicated times. HA-Cdc25A was immunoprecipitated, separated by SDS-PAGE, and analyzed by autoradiography. An asterisk indicates a crossreacting protein.
C: Quantitation of data from cycloheximide pulse-chase experiments with wild-type or the indicated mutants of HA-Cdc25A performed as in **A**. The plotted values show the levels of remaining HA-Cdc25A proteins measured between 30 min and 1 hr after IR and addition of cycloheximide, relative to the levels in lysates from untreated cells. The results shown are an average of 3–4 independent experiments.

selectively targeted Chk1 and Chk2, respectively, in live cells by RNAi. Indeed, the genetic downregulation of either Chk1 or Chk2 also leads to a decrease of IR-induced phosphorylation of Cdc25A on S123 (Figure 5D). Together, these data suggest that the turnover of Cdc25A is regulated by a two-step mechanism. First, in undamaged cells, Chk1 alone determines the basal degradation rate of Cdc25A by continuous phosphorylation of S123 and three other sites identified in this study. Second, after IR exposure, the rate of phosphate incorporation into at least a subset of these residues increases due to a combination of the ongoing activities of Chk1- and IR-induced Chk2 that jointly promote the accelerated turnover of Cdc25A.

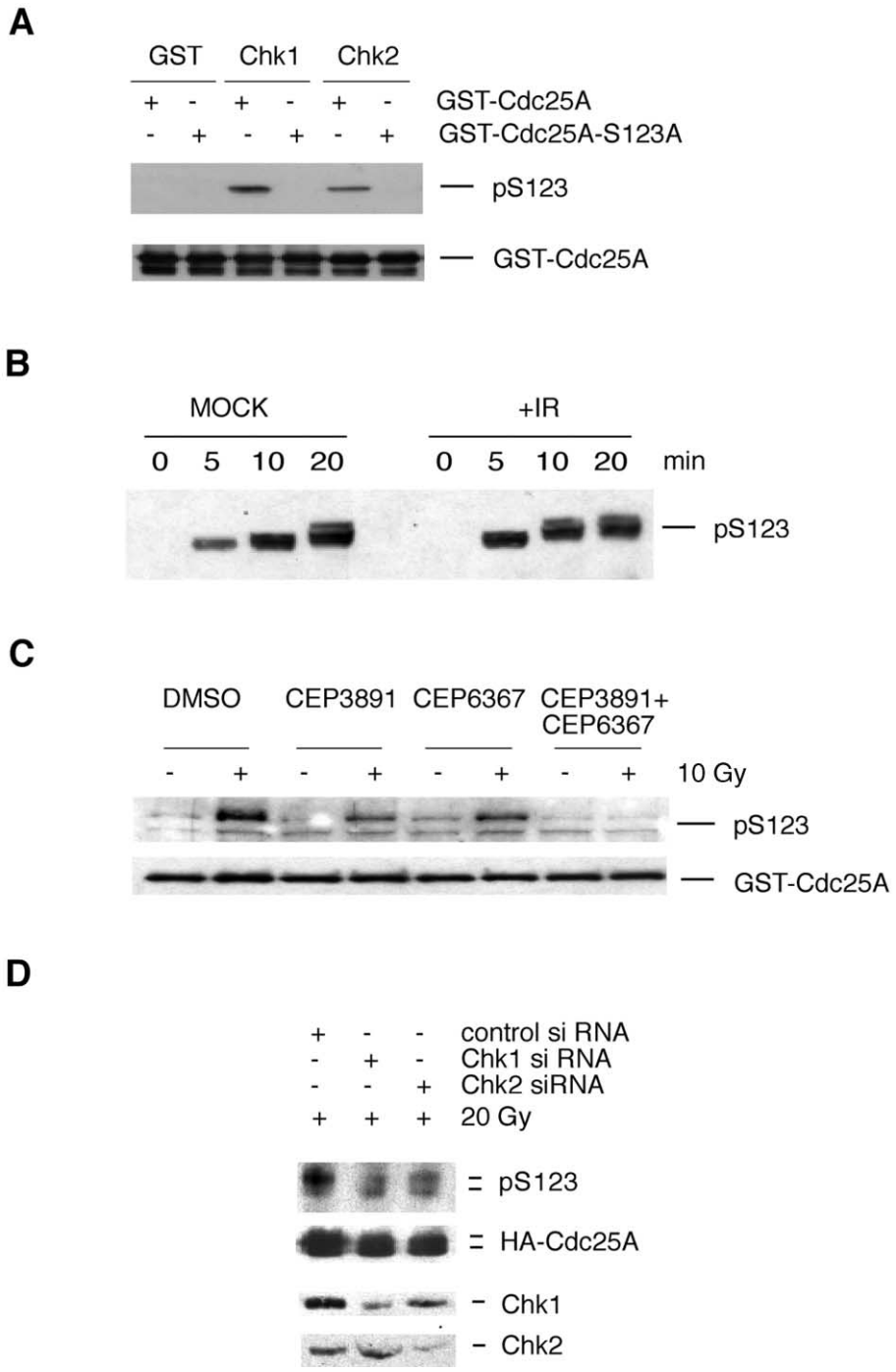

Figure 5. Accelerated rate of phosphorylation of S123 after IR

A: An in vitro kinase assay using recombinant Chk1 and Chk2 to phosphorylate wild-type GST-Cdc25A or GST-Cdc25A S123A was followed by blotting with the phospho S123 antibody (pS123) to confirm its specificity for S123-phosphorylated Cdc25A.

B: The kinetics of phosphorylation on S123 were assayed using extracts prepared from nonirradiated and irradiated U2-OS cells (10 Gy, 1 hr). In vitro kinase assays were performed using GST-Cdc25A as a substrate for the indicated times, followed by Western blotting with the pS123-phosphospecific antibody.

C: The kinetics of S123 phosphorylation were assayed as in **B** and evaluated 10 min after initiation of the kinase reaction. The reactions were supplemented either by DMSO (control reaction) or with Chk1 inhibitor (CEP-3891), Chk2 inhibitor (CEP-6367), or both inhibitors combined, respectively (see further characterization of the CEP drugs in Supplemental Figure S1 and Supplemental Tables S1 and S2 at http://www.cancercell.org/cgi/content/full/3/3/247/DC1).

D: Phosphorylation on S123 in vivo requires Chk1 and Chk2. U2OS cells conditionally expressing HA-Cdc25A were either nontransfected (left two lanes) or transfected with control, Chk1, or Chk2 siRNA (right 3 lanes). At 30 hr after transfection, expression of HA-Cdc25A was induced by removal of tetracyclin for 4 hr, followed by irradiation and incubation for 1 hr in the presence of the proteasome inhibitor MG132 to prevent elimination of the phosphorylated unstable Cdc25A protein. Immunoprecipitations using an antibody to the HA-tag followed by Western blotting with the pS123 and HA antibodies are shown. Western blotting of Chk1 and Chk2 demonstrate efficient downregulation of both proteins by the siRNA treatments.

Chk1 is required to prevent RDS in cells exposed to IR

Since inhibition of Chk1 abrogates IR-induced downregulation of Cdc25A, we asked whether inhibition of Chk1 would affect the S phase checkpoint and cause RDS. Indeed, addition of the Chk1 inhibitors UCN-01 or CEP-3891 prior to irradiation partly rescued the inhibition of DNA synthesis after IR (Figure 6A, left panel). Consistently, HeLa cells transfected with Chk1 siRNA showed less inhibition of DNA synthesis in response to IR than cells treated with control siRNA (Figure 6A, middle panel). Treatment with Chk2-specific siRNA also caused RDS (Figure 6A, middle panel), consistent with the previously identified role of Chk2 in Cdc25A degradation after IR (Falck et al., 2001). Combination of Chk1 and Chk2 siRNA did not lead to further increase in the extent of RDS achieved by single siRNA treatments (data not shown). This may indicate that in the absence of Chk1 (which would lead to stabilization of Cdc25A even without IR), Chk2 alone is insufficient to stimulate the S phase checkpoint. In any case, the siRNA data do support our conclusion that Chk1 is an integral component of the rapid IR-induced S phase checkpoint.

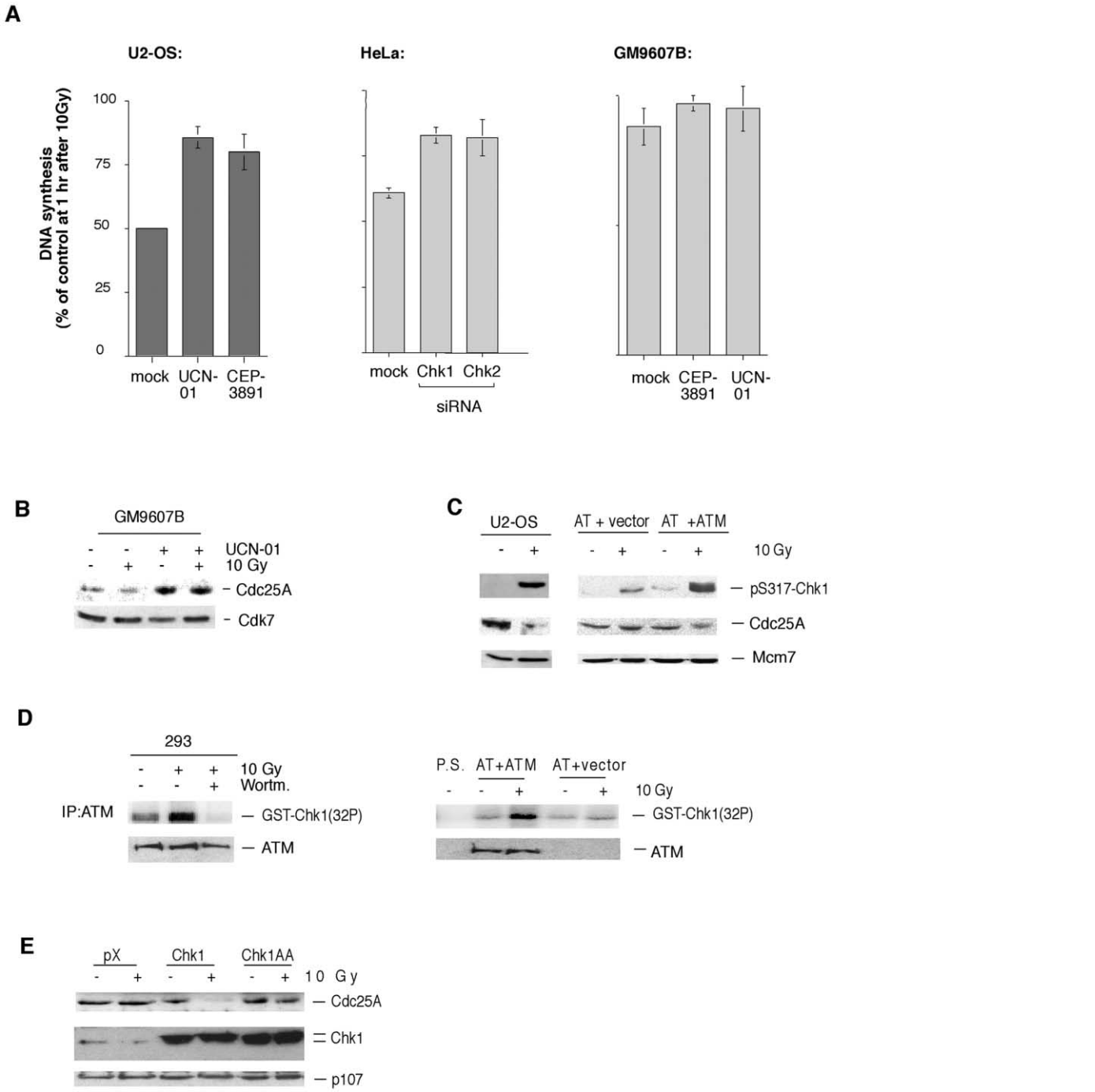

Figure 6. Chk1 is required for the rapid ATM-dependent S phase checkpoint after IR

A: Inhibition of Chk1 abrogates IR-induced inhibition of DNA synthesis. U2-OS cells treated with Chk1 inhibitors UCN-01 or CEP-3891 (left panel), or HeLa cells transfected with Chk1 or Chk2 siRNA (middle panel), show reduced downregulation of DNA synthesis at 1 hr after IR. No significant effect is seen by addition of UCN-01 or CEP-3891 to AT fibroblasts (right panel). DNA synthesis relative to nonirradiated cells is shown for each treatment, respectively.

B: Basal level of Cdc25A is increased in AT-cells after Chk1 inhibition. ATM-deficient GM9607B cells were treated with UCN-01 and exposed to 10 Gy of IR as indicated. One hr later, the cell lysates were analyzed by Western blotting for the levels of endogenous Cdc25A and Cdk7 (loading marker).

C: Phosphorylation of Chk1 after IR is ATM-dependent and correlates with Cdc25A degradation. Western blots with antibodies specific to Chk1 phosphorylated at serine 317 and Cdc25A are shown for U2-OS cells and AT cells (AT22IJE-T) transfected with control vector or recombinant ATM. Extracts were prepared at 1 hr after IR. Mcm7 protein is a loading marker.

D: IR-induced ATM directly phosphorylates Chk1. ATM was immunoprecipitated from extracts prepared from 293 cells (left panel) or AT or ATM-reconstituted AT cells (right panel) at 1 hr after IR. Where indicated, 5 μM of the ATM-inhibiting drug Wortmannin was added to show the specificity of the kinase reaction.

E: ATM phosphorylation of Chk1 is required for efficient IR-induced downregulation of Cdc25A. HeLa cells were transfected with expression plasmids containing no insert (pX), Flag-Chk1, or Flag-Chk1AA (S317A, S345A). Each reaction was supplied with an expression plasmid for CD20 transfection marker. Subsequently, cells were transfected with Chk1 siRNA to deplete endogenous Chk1. After additional 30 hr, the cells were irradiated with 10 Gy for 1 hr, harvested, sorted via CD20-coated magnetic beads, and processed for Western blotting. The assay was reproduced three times with consistent results.

ATM directly targets Chk1 to regulate the IR-induced S phase checkpoint

ATM-deficient (AT) cells are defective in radiation-induced S phase checkpoint and display RDS (Painter and Young, 1980). To directly assess the relationship between ATM and Chk1 in regulating the S phase checkpoint, we examined the effects of Chk1 inhibition in AT cells. No significant effects were seen on DNA synthesis after IR by addition of UCN-01 or CEP-3891 to AT fibroblasts (Figure 6A, right panel). Consistent with the RDS phenotype, Cdc25A is not degraded efficiently in AT cells after IR (Falck et al., 2001 and Figure 6B). However, addition of UCN-01 caused increased Cdc25A protein levels in unstressed A-T cells (Figure 6B, lanes 3 and 4). These data indicate that while Chk1-mediated control over the basal turnover of Cdc25A is ATM-independent, the accelerated destruction of Cdc25A after IR may depend on a functional link between ATM and Chk1. Whether ATM and Chk1 operate along one linear pathway has been tested in the following experiments.

In response to UV light Chk1 kinase activity increases upon phosphorylation on serines 317 and 345 likely mediated by the upstream kinase ATR (Guo et al., 2000; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). The IR-induced effects on Chk1 are less clear. Using the available phosphospecific antibodies and different cell types, we observed that Chk1 was phosphorylated on S317 and S345 rapidly after IR in a dose-dependent manner (Figure 6C and data not shown). To test whether IR-induced phosphorylation of Chk1 is ATM-dependent, we used AT cells transfected with control vector or stably reconstituted with ATM (Ziv et al., 1997). Strikingly, the control AT cells showed impaired phosphorylation of Chk1 on S317 after IR, and reconstitution with ATM rescued the IR-induced phosphorylation on S317 (Figure 6C) and S345 (data not shown). As expected, this was accompanied by a restoration of Cdc25A degradation in the reconstituted cells after irradiation (Figure 6C). Consistently, endogenous ATM immunoprecipitated from 293 cells (Figure 6D, left panel) and overexpressed ATM immunopurified from ATM-reconstituted A-T cells (Figure 6D, right panel) efficiently phosphorylated recombinant Chk1. This phosphorylation included S317 and S345 (Supplemental Figure S5 at http://www.cancercell.org/cgi/content/full/3/3/247/DC1 and data not shown). Finally, we tested the relevance of the ATM-mediated phosphorylation of Chk1 by reconstitution of Chk1 expression in cells deprived of endogenous Chk1 by RNAi. Remarkably, while the wild-type Chk1 efficiently rescued the IR-induced accelerated turnover of Cdc25A in cells deprived of endogenous Chk1, the Chk1 allele where both S317 and S345 were substituted to alanines was reproducibly less efficient (Figure 6E). This occurred despite the associated kinase activities of wild-type and mutant Chk1 were similar (data not shown). Collectively, these data suggest that in addition to its role in maintaining the physiological turnover of Cdc25A, Chk1 actively contributes to a full-scale destruction of Cdc25A after exposure to IR, and that the latter (but not the former) Chk1 function operates in an ATM-dependent manner.

Discussion

Regulation of the basal turnover of Cdc25A: A novel function of Chk1 in cell cycle coordination and maintenance of genome integrity

A homozygous disruption of Chk1 in mice causes severe checkpoint defects in ES cells and results in peri-implantation embryonic lethality (Liu et al., 2000; Takai et al., 2000). This indicates that in metazoans, Chk1 controls essential cell cycle events irrespective of DNA damage and/or forced replicative stress. However, specific molecular mechanisms that would mediate the dramatic loss of viability of mammalian cells lacking Chk1 have not been characterized. Our results together with a recent report by Piwnica-Worms and colleagues (Zhao et al., 2002) begin to shed light on this important issue by showing that transient downregulation of Chk1 by inhibitory drugs or RNAi was sufficient to elevate endogenous Cdc25A to supraphysiological levels. Overexpression of Cdc25A has dramatic consequences for cell cycle progression, including unscheduled DNA replication and cell death (Molinari et al., 2000; Vigo et al., 1999). In addition, excessive accumulation of Cdc25A was found in diverse human malignancies (Cangi et al., 2000; Wu et al., 1998), raising a possibility that it can contribute to genetic instability (Galaktionov et al., 1995). Finally, unscheduled expression of cyclin E, the prime target of Cdc25A linked to origin firing (Blomberg and Hoffmann, 1999), was shown to induce gross chromosomal aberrations (Spruck et al., 1999). Based on these findings, our present results, and the mounting evidence that Chk1 itself is subverted by mutations in diverse human malignancies (Bertoni et al., 1999; Menoyo et al., 2001; Vassileva et al., 2002), we propose that one way mammalian Chk1 protects genome integrity may be through counterbalancing the Cdc25A synthesis during physiological S phase by promoting its rapid turnover, thereby keeping the proreplicative activity of cyclin E/Cdk2 within the physiological borders. Consistently, we could clearly show that cyclin E/Cdk2 kinase activity was upregulated when Cdc25A was stabilized through Chk1 inhibition (see Figure 1).

Since Cdc25A accumulates at the G1/S transition, the model of a critical role of Chk1 in preventing overaccumulation of Cdc25A in an unperturbed cell cycle predicts that Chk1 is periodically activated in every S phase. While the majority of studies refer to Chk1 as a DNA damage- and/or replication stress-inducible kinase, a recent report indeed provided evidence for a close correlation of Chk1 accumulation and increase of its kinase activity without any external DNA stress (Kaneko et al., 1999). Our data showing that both chemical and genetic inhibition of Chk1 decreased the Chk1 kinase activity in nonirradiated cells (see Figure 1) support such a scenario. We suggest that following its E2F-mediated induction at the G1/S transition (Gottifredi et al., 2001; Ren et al., 2002), Chk1 becomes active irrespective of DNA damage, possibly by signals transmitted from the generation and/or processing of the physiological replication structures. Interestingly, Cdc25A expression is also transcriptionally regulated by E2F (Vigo et al., 1999), indicating that the complex E2F-regulated S phase-promoting program includes coordinated induction of a powerful inducer of DNA replication (Cdc25A) with an immediate means (Chk1) to limit this activity, thereby preventing excessive and unscheduled firing of replication origins (Figure 7A).

Requirement of Chk1 for the IR-induced accelerated proteolysis of Cdc25A

We could demonstrate that Chk1 is required for IR-induced downregulation of Cdc25A and that ablation of Chk1 function leads to RDS. While this paper was under review, similar observations have been reported (Zhao et al., 2002). One way that Chk1 participates in the IR-induced S phase checkpoint may

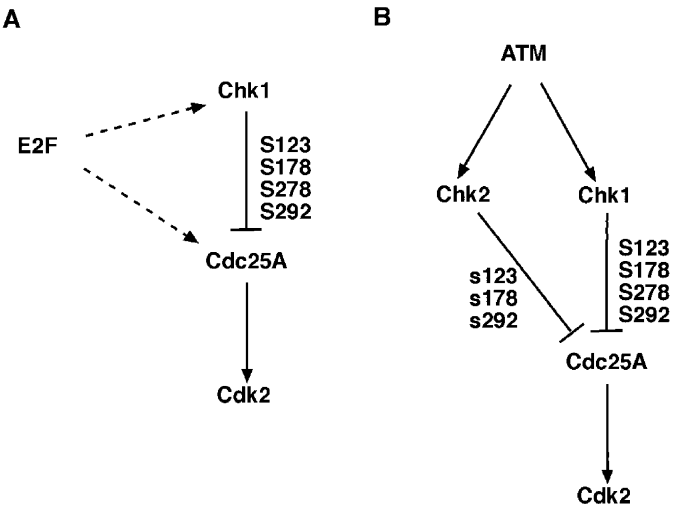

Figure 7. Model for Chk1-mediated regulation of Cdc25A and the S phase checkpoint

A: Chk1 controls Cdc25A in the absence of IR. Both Chk1 and Cdc25A are positively regulated by the E2F transcription factor when cells enter S phase. Chk1 promotes basal Cdc25A protein turnover by direct phosphorylations on multiple Cdc25A residues (S123, S178, S278, and S292). Cdc25A turnover buffers the Cdc25A phosphatase activity, and thereby its capability to activate cyclin E/A-cdk2 complexes that regulate S phase entry and progression, respectively.

B: Chk1 is required for radiation-induced downregulation of Cdc25A. After IR, Cdc25A protein turnover accelerates due to an increased phosphorylation on multiple Cdc25A residues that overlap with those constitutively phosphorylated by Chk1 in the absence of IR (see **A**). The increased phosphorylation of S123, S178, S278, and S292 is achieved by a cooperative action of ATM-regulated Chk1 and Chk2 kinases. This results in a rapid IR-induced degradation of Cdc25A, which ultimately leads to inhibition of Cdk2 and suppression of initiation of DNA replication.

again relate to its role in controlling the basal turnover of Cdc25A. As discussed above, the Cdc25A protein is unstable in normal S phase due to ongoing proteolysis, which is controlled by Chk1 via direct phosphorylation of at least 4 residues. We propose that such Chk1-mediated basal control of Cdc25A turnover is a necessary prerequisite to accommodate any IR-induced signals to accelerate the proteolysis of Cdc25A. Such a scenario is consistent with our observation that IR exposure did not involve new phosphorylation sites, but rather increased the rate of phosphorylation on the same residue of Cdc25A that was already phosphorylated by Chk1 in nonirradiated cells (see Figure 5).

Furthermore, we showed that exposure to IR induced ATM-dependent posttranslational modifications of Chk1, including phosphorylation of serines 317 and 345. Together with the ability of immunopurified ATM to phosphorylate Chk1 and the fact that mutation of serines 317 and 345 impaired the ability of Chk1 to promote the IR-induced degradation of Cdc25A, these results strongly suggest that regulation of the IR-induced S phase checkpoint relies on the direct ATM-Chk1 functional interplay.

Such posttranslational modifications of Chk1 might reflect important processes required for optimal performance of the Chk1 kinase (although they may not necessarily alter the Chk1 kinase activity to a great extent). Translated specifically to the Chk1-Cdc25A interplay, IR-induced Chk1 modifications may help protect this vital cellular factor (in unstressed cells, Chk1 is an unstable protein [Lukas et al., 2001]), or secure and/or enhance the ongoing access of Chk1 to Cdc25A by recruiting essential cofactor, such as the recently identified claspin (Kumagai and Dunphy, 2000).

Primary MEFs isolated from mice with a homozygously deleted Chk2 gene did not undergo RDS, and it has been reasoned that Chk1 may compensate for Chk2 in phosphorylation of Cdc25A (Hirao et al., 2002). Our results provide direct mechanistic evidence that this indeed might be the case, since Chk1 is an essential ATM-regulated component of the IR-induced signaling to accelerate proteolysis of Cdc25A. On the other hand, in response to IR, the ATM-dependent phosphorylations of Chk1 are less pronounced compared to the entirely ATR-dependent checkpoint responses such as stalled replication forks and/or the UV-type of DNA damage (Liu et al., 2000; Zhao and Piwnica-Worms, 2001; and our unpublished data). Thus, while ATR-mediated checkpoint responses appear to depend solely on Chk1 to switch the Cdc25A proteolysis to the accelerated mode, the IR-induced DNA damage in somatic cells requires a cooperative action of other ATM-induced factors such as Chk2 (Figure 7B), previously identified in our laboratory as one mediator of the timely Cdc25A degradation in response to IR (Falck et al., 2001). This "cooperative" model has been further strengthened by several new observations in our present study. First, the selective siRNA downregulation of Chk1 and Chk2, respectively, impaired the IR-induced Cdc25A phosphorylation. Second, the same genetic ablation of either kinase produced a RDS phenotype. Third, the concomitant chemical inhibition of both kinases in extracts from irradiated cells was required for a more complete inhibition of the ability of these lysates to phosphorylate Cdc25A in vitro (see Figures 5 and 6). The proposed cooperative action of ATM-activated Chk2 and ATM-modified Chk1 might be instrumental to rapidly reach and maintain the threshold of the IR-induced cell defense mechanism that would be sufficiently high to prevent unscheduled origin firing and other events potentially priming for acute genetic instability.

Amplification of basic events associated with DNA replication: An effective means to avoid RDS without irreversible disruption of cell cycle progression

Provided the extent of DNA damage is not beyond repair, the main purpose of cell cycle checkpoints appears to be protecting the genomic integrity and resumption of cell cycle progression. Hence, it would be dangerous, especially if the intra-S-phase checkpoint included powerful but irreversible means to arrest the cell cycle such as complete destruction of cell cycle regulators that mark the main unidirectional cell cycle decisions. It rather seems that a fine modulation of the ongoing processes, such as the dynamics of Cdc25A phosphorylation and proteolysis uncovered here, might be the desirable means to slow down the pace of DNA replication and gain time for a productive repair of the lesion.

We showed that Chk1 is required for at least one essential event (regulation of basal turnover of Cdc25A) accompanying every physiological S phase. Regarding the IR-induced S phase checkpoint, we propose that the ATM signaling to Cdc25A may not need to initiate damage-specific pathways, but rather modulate the kinetics of the existing, Chk1-controlled S phase

processes. In such a model, ATM-downstream (and strictly damage-inducible) events including IR-induced modification of Chk1 itself (this study) and activation of Chk2 (Falck et al., 2001) would act as modifiers, nonessential to support the imminent requirement for cell survival but important especially under conditions when cells encounter increasing demand to cope with stress and more vigorous replication activity. Active involvement of Chk1 in the intra-S-phase cell cycle response to IR-induced genotoxic stress, uncovered in this study, is consistent with candidacy of Chk1 for a tumor suppressor, a notion supported by mutations of Chk1 detected in diverse human malignancies (Bertoni et al., 1999; Menoyo et al., 2001; Vassileva et al., 2002).

Experimental procedures

Cell lines and siRNA treatment

Human U2-OS osteosarcoma, 293 kidney epithelial, and HeLa cervical carcinoma cell lines, U2-OS derivative conditionally expressing HA-Cdc25A (Mailand et al., 2000), and AT fibroblasts GM09607 (Coriell Cell Repositories) were grown in DMEM medium with 10% fetal bovine serum. Human AT fibroblasts AT22IJE stably transfected with YZ5 ATM cDNA (Ziv et al., 1997) were grown in the presence of 100 μg/ml hygromycin.

For siRNA-mediated ablation of Chk1, HeLa cells were transfected with the following oligonucleotide sequences: 5′-UCGUGAGCGUUUGUUGAA CdTdT-3′ (Chk1 siRNA) and 5′-GAAGCAGUCGCAGUGAAGAdTdT-3′ (control siRNA), by OligofectAMINE™ Reagent (Invitrogen). The control siRNA contains a nonfunctional Chk1 target sequence that is unable to downregulate Chk1. Chk2 siRNA were directed against the sequence 5′-AAGAACCU GAGGACCAAGAACdTdT-3′. Experiments with siRNA-transfected HeLa cells were performed at 30–35 hr after transfection. Reconstitution of Chk1 expression in siRNA treated HeLa cells was done by mutating 4 residues in the siRNA target sequence of Flag-tagged Chk1 (TTGTTG was changed to CTCCTC) that changed the nucleotide but not the amino acid sequence of Chk1. Cells were first cotransfected with expression plasmids for Chk1 and the CD20 transfection marker by the calcium-phosphate method, followed by siRNA transfection. 30 hr after siRNA treatment, cells were sorted with anti-CD20 magnetic beads and lysed (Sørensen et al., 2001).

Drugs and irradiation

The CEP-3891 Chk1 inhibitor and the CEP-6367 Chk2 inhibitor were provided by CEPHALON Inc. UCN-01 was a gift from R.J. Schultz (Drug Synthesis & Chemical Branch, National Cancer Institute). The CEPHALON inhibitors were used at a concentration of 500 nM, and UCN-01 was used at 300 nM. Wortmannin was purchased from Calbiochem. Ionizing radiation was delivered by X-ray generator (RT100, Phillips Medico; 100 kV, 8 mA, doserate 0.92 Gy/min).

Antibodies and immunochemistry

Immunoblotting, immunoprecipitation, Cdc25A phosphatase activity, and in vitro kinase assays have been described (Mailand et al., 2000). ATM kinase assays were performed as described (Kim et al., 1999) using anti-Flag antibody (M2 from Sigma) or two ATM antibodies (PC116 from Oncogene and NB100-104 from Novus Biologicals). Purified GST-Chk1 (270–476 aa) was used as a substrate. We generated a mouse antibody to Chk1 (DCS-310), used here for immunoblotting, by standard hybridoma technology (Harlow and Lane, 1988). For immunoprecipitation of Chk1, we used sheep anti-Chk1 from Upstate Biotechnology. Phospho-Chk1 antibodies (S317 and S345) were purchased from Cell Signaling. A rabbit antibody specific to phosphorylated Cdc25A on S123 was supplied by GlaxoSmithKline Pharmaceuticals. Antibodies to HA-tag (12CA5), Cdc25A (DCS-124), Chk2 (DCS-270 and DCS-273), cyclin E (HE172), MCM-7 (DCS-141), and Cdk7 (MO-1) have been described (Falck et al., 2001). Antibodies to Cdc25A (F-6), cyclin A (sc751), and cyclin B (sc245) were from Santa Cruz Biotechnology.

Inhibition of DNA synthesis

Cells were labeled for 24 hr with 20 nCi/ml [^{14}C]Thymidine, followed by another 24 hr incubation in nonradioactive medium. Cells were then irradiated (0 Gy or 10 Gy), and [^{3}H]Thymidine (2.5 μCi/ml, 2Ci/mmol) was added at 1 hr after irradiation. At 20 min after addition of [^{3}H]Thymidine, the cells were fixed in 70% methanol, washed in PBS, and radioactivity was measured in a liquid scintillation counter. The ratios of [^{3}H]/ [^{14}C] were calculated and DNA synthesis after irradiation was expressed as a percentage of unirradiated values. This was done to compensate for the differential impact of the siRNA on basal DNA replication in unirradiated cells (Chk2-directed siRNA inhibited the basal rate of DNA synthesis to a larger extent than Chk1 or control siRNA, data not shown).

Cycloheximide and [^{35}S]-methionine pulse-chase

Cells were transfected by the calcium-phosphate method for 12 hr. After an additional 7 hr, a cycloheximide chase was performed as described previously (Falck et al., 2001). For [^{35}S]-methionine pulse-chase, cells were washed 3 times with methionine-free DMEM at 7 hr after transfection, followed by labeling for 0.5 hr with 100 μCi/mL [^{35}S]-methionine (SJQ 0047; Amersham). Labeling was done in methionine-free DMEM with 10% dialyzed serum. Cell lysates were prepared at the indicated time points and processed for immunoprecipitation.

Orthophosphate labeling and two-dimensional electrophoresis

Cells were labeled for 2 hr in phosphate-free DMEM with 20 mM Hepes (pH 7.2), 10% dialyzed serum, and 0.5 mCi/mL [^{32}P]orthophosphate (PBS43; Amersham). In some experiments, the proteasome inhibitor MG132 (5 μM) was added. Cells were lysed with RIPA-buffer (Harlow and Lane, 1988) and processed for immunoprecipitation. Two-dimensional phosphopeptide mapping and phosphoamino acid analysis were performed as described (Boyle et al., 1991). Tryptic phosphopeptides were separated on thin-layer cellulose plates using a Hunter Thin Layer Electrophoresis System (HTLE-7000; 2000 V for 25 min), followed by chromatography in the second dimension (isobuturic acid buffer).

Elution of peptides for Edman degradation

Eluted phosphopeptides were coupled to Sequelon-AA membrane by use of carbodiimide coupling, as described by the manufacturer. Edman degradation was performed using an Applied Biosystems gas phase sequencer (Model 477A) as described (Blume-Jensen et al., 1995).

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