Degradation of Cdt1 during S Phase Is Skp2-independent and Is Required for Efficient Progression of Mammalian Cells through S Phase*

Received for publication, February 2, 2005, and in revised form, April 20, 2005
Published, JBC Papers in Press, April 25, 2005, DOI 10.1074/jbc.M501208200

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Previous reports have shown that the N terminus of Cdt1 is required for its degradation during S phase (Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003) J. Biol. Chem. 278, 30854–30858; Nishitani, H., Lygerou, Z., and Nishimoto, T. (2004) J. Biol. Chem. 279, 30807–30816). The stabilization was attributed to deletion of the cyclin binding motif (Cy motif), which is required for its phosphorylation by cyclin-dependent kinases. Phosphorylated Cdt1 is subsequently recognized by the F-box protein Skp2 and targeted for proteasomal mediated degradation. Using phosphopeptide mapping and mutagenesis studies, we found that threonine 29 within the N terminus of Cdt1 is phosphorylated by Cdk2 and required for interaction with Skp2. However, threonine 29 and the Cy motif are not necessary for proteolysis of Cdt1 during S phase. Mutants of Cdt1 that do not stably associate with Skp2 or cyclins are still degraded in S phase to the same extent as wild type Cdt1, indicating that other determinants within the N terminus of Cdt1 are required for degrading Cdt1. We localized the region necessary for Cdt1 degradation to the first 32 residues. Overexpression of stable forms of Cdt1 significantly delayed entry into and completion of S phase, suggesting that failure to degrade Cdt1 prevents normal progression through S phase. In contrast, Cdt1 mutants that fail to interact with Skp2 and cyclins progress through S phase with similar kinetics as wild type Cdt1 but stimulate the re-replication caused by overexpressing Cdt1. Therefore, a Skp2-independent pathway that requires the N-terminal 32 residues of Cdt1 is critical for the degradation of Cdt1 in S phase, and this degradation is necessary for the optimum progression of cells through S phase.

* This work was supported in part by National Institutes of Health Grant CA89406 (to A. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Supported by a Howard Hughes Medical Institute predoctoral fellowship and National Institutes of Health Grant CA89406.
¶ Supported by National Institutes of Health Grant CA90281.
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1 The abbreviations used are: pre-RC, pre-replicative complex; CDK, cyclin-dependent kinase; Cy motif, cyclin binding motif; FACS, fluorescence-activated cell sorter; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein; ORC, origin recognition complex; WT, wild type.

During G1 phase of the cell cycle, large multi-protein complexes referred to as pre-replicative complexes (pre-RCs) assemble at replication origins throughout the genome, “licensing” the DNA for replication in S phase (1, 2). Pre-RCs form in a stepwise fashion, beginning with the six-subunit origin recognition complex (ORC) that binds to origins and recruits Cdc6 and Cdt1, which are, in turn, both required for the subsequent recruitment of the replicative helicase Mcm2-7 (3). Transition to S phase is accompanied by a dramatic increase in the levels of cyclin-dependent kinases (Cdks), which activate pre-RCs by mediating recruitment of the replicative polymerases that begin DNA synthesis. High Cdk activity during S phase also inhibits the formation of new pre-RCs. Therefore, origins are prevented from becoming re-replicated until the next M/G1 transition, at which point cyclins are rapidly degraded and pre-RCs are again allowed to assemble. Consistent with this model, perturbation of Cdk levels such as inhibiting Cdk activity in G2 prevents Cdt1 degradation during G1, when pre-RCs are formed and degraded in S phase when pre-RCs are fired and disassembled (8, 13). Regulation is primarily at the protein level, as mRNA levels of Cdt1 remain constant throughout the cell cycle (13). It was recently shown that Cdt1 is phosphorylated in vivo and that its phosphorylation is required for interaction with the F-box protein Skp2 (14). Skp2 is a component of the SCF complex, which functions as an E3 ubiquitin ligase. The SCF<sub>Skp2</sub> complex has been implicated in the ubiquitination and ensuing proteasomal degradation of several cell cycle proteins at the G1/S transition, with the Skp2 subunit specifically recognizing phosphorylated substrates. Subsequently, it was shown that Cdt1 is phosphorylated by Cdk2, suggesting that Cdt1 degradation is mediated through Cdk phosphorylation, which targets it for ubiquitination by SCF<sub>Skp2</sub> (15–17). However, definitive evidence for this molecular mechanism is still lacking. Skp2<sup>−/−</sup> murine cells have been reported to show no difference in the expression of Cdt1 protein compared with that of wild type (WT) cells (18). Although transient silencing of Skp2 by RNA interference increases Cdt1 steady state levels (14), reducing Skp2 levels also has been shown to prevent entry into S phase by stabilizing p27 (19–21). Because Cdt1 is stable in G1 phase, altering cell cycle progression could explain changes in Cdt1 stability. Additional

*This paper is available online at http://www.jbc.org
evidence arguing against Cdk-mediated Skp2 degradation includes recent experiments in Xenopus demonstrating a Cdk-independent mechanism for degrading Cdt1 after origin firing (22).

Based on these conflicting data, we decided to more closely examine the significance of the interactions between Cdt1, Skp2, and Cdk phosphorylation in mammalian cells by identifying the phosphosites on Cdt1 required for Skp2 binding. By using point mutants of Cdt1, we avoid the nonspecific effects generated by manipulating Skp2 levels. Although we confirm that the N terminus of Cdt1 is required for its degradation, mutants of Cdt1 that do not interact with Skp2 and cyclins are still degraded during S phase, suggesting the presence of Skp2-independent pathways for Cdt1 destruction in S phase. These mutants stimulate the re-expansion of Cdt1, suggesting that phosphorylation by cyclin/Cdk2 and/or interaction with Skp2 has a negative effect on Cdt1 activity independent of proteolysis. In contrast, expression of Cdt1 mutants that are stable during S phase cause a delayed entry into and completion of S phase, suggesting that failure to degrade Cdt1 prevents proper progression through S phase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections—**HeLa and 293T cells were grown as adherent monolayers in Dulbecco’s modified Eagle’s medium supplemented with 10% donor calf serum. Transfections were performed using Lipofectamine 2000 (Invitrogen) reagent or electroporation for chromatin fractionation experiments using the Amaxa Nucleofector system according to the manufacturer's instructions. For FACS analyses, a plasmid expressing farnesylated green fluorescent protein, pEGFP (Clontech), was cotransfected with HA-tagged wild type Cdt1 and Cdt1(S31A), blocked with nocodazole, and processed as described for panel A. Immunoblotting with anti-cyclin A was used as a marker for S phase, and anti-α-tubulin was used as a loading control.

**Plasmids—**The plasmids for expression of recombinant cyclin A/Cdk2 in *Escherichia coli* were described previously (8, 23). Mammalian expression plasmids for HA-tagged Cdt1 were constructed by subcloning Cdt1 from pGEX-5X1 into pAHP using the EcoRI and NotI restriction sites. For plasmids expressing GST-tagged Cdt1 in mammalian cells, Cdt1 was amplified by PCR using primers engineered with restriction sites and cloned into the SpeI and NotI sites of pEBG. Mutations in Cdt1 were created using the Stratagene QuickChange protocol with the following oligonucleotides pairs: 5′-AGGCGACGGCCCGCCGTCGGTGGAC-3′ and 5′-AGGCCACC GGCCCGCCGTCGGTGGAC-3′; 5′-AGGCGACGGCCCGCGCCGTCGGTGGAC-3′ and 5′-AGGCCACC GGCCCGCCGTCGGTGGAC-3′; and 5′-AGGCGACGGCCCGCCGTCGGTGGAC-3′ and 5′-AGGCCACC GGCCCGCCGTCGGTGGAC-3′. The antibodies used were Cdt1 (8), cyclin A (Santa Cruz Biotechnology), 12CA5 anti-HA (24), Skp2 (Santa Cruz Biotechnology), Mcm7 (Santa Cruz Biotechnology), Cdc45 (26), geminin (8), and α-tubulin (Sigma). Immunoprecipitation and immunoblotting were performed according to standard procedures. FACS Analysis—Cells harvested from tigroxin were fixed with 70% ethanol and incubated with propidium-buffered saline containing 5 μg/ml propidium iodide, 5 μg/ml RNase, and 0.1% Triton X-100 for 30 min at room temperature. At least 10,000 cells were counted per sample. FACS analyses were performed using a BD Biosciences flow cytometer and CellQuest™ software.

**RESULTS**

**The N Terminus of Cdt1 Regulates Its Stability during S Phase—**Within the N terminus of Cdt1 are several putative Cdk phosphorylation sites as well as the previously identified cyclin binding motif (Cy motif) located at residues 67–71 (15, 16). Therefore, we constructed an N-terminal truncation mutant of Cdt1 by deleting the first 93 amino acids. We also needed to append an SV40 nuclear localization signal in order to properly localize Cdt1(S31A) to the nucleus (17). HeLa cells were co-transfected with WT Cdt1 and Cdt1(S31A) and synchronized in mitosis with nocodazole. To collect cells in G1, cells were harvested 8 h after release from nocodazole. Cells in S phase were obtained by releasing cells from nocodazole into hydroxyurea. FACS analysis and immunoblotting with anti-cyclin A were used to confirm the cell cycle phase. Fig. 1B shows that WT Cdt1 is stable in G1 and degraded in S phase, identical to what is seen with endogenous Cdt1 (Fig. 1A). These results also indicate that we are not overwhelming the degradative machinery by overexpressing Cdt1. In contrast, Cdt1(S31A) is stable in S phase (Fig. 1B). Therefore, the first 93 amino acids of Cdt1 are necessary for its degradation during S phase. Recent studies report that the N terminus of Cdt1 is not required for its licensing function, indicating that deleting the N terminus of Cdt1 does not result in a completely misfolded and nonfunctional protein (27, 28).

Mapping the Phosphorylation Sites of Cdt1 in Vivo—Because phosphorylation of Cdt1 is required for associ-

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*FIG. 1. The N terminus of Cdt1 regulates its stability. A. HeLa cells were released from a nocodazole block and separated evenly into two separate dishes. One was harvested 8 h after release and represents cells in G1 phase of the cell cycle. Hydroxyurea was added to the second dish after release and harvested after 14 h to obtain cells in S phase. Lysates were immunoblotted with anti-Cdt1 and anti-cyclin A. The presence of cyclin A was used as a marker for S phase. The asterisk represents a nonspecific band used as a loading control. B. HeLa cells were co-transfected with HA-tagged wild type Cdt1 and Cdt1(S31A), blocked with nocodazole, and processed as described for panel A. Immunoblotting with anti-cyclin A was used as a marker for S phase, and anti-α-tubulin was used as a loading control.*
corresponding to Cdt1 was excised and processed as described for untransfected sample used to identify nonspecific bands. The band phosphorylated.

Asterisks indicate residues that were phosphorylated. B, GST-tagged Cdt1-(1–93) was purified from 293T cells after transfection and resolved by SDS-PAGE followed by Coomassie Blue staining. The left lane represents an untransfected sample used to identify nonspecific bands. The band corresponding to Cdt1 was excised and processed as described for panel A. The bottom section shows the amino acid sequence for the first 93 residues of Cdt1, with the peptides recovered by mass spectrometry highlighted in boldface type. Asterisks indicate residues that were phosphorylated.

FIG. 2. Phosphopeptide mapping of Cdt1 in vitro and in vivo. A, purified recombinant Cdt1 was separated by SDS-PAGE after being phosphorylated in vitro using recombinant cyclin A/Cdk2. After staining with Coomassie Blue, the bands corresponding to phosphorylated Cdt1 (Cdt1-P) were excised from the gel and analyzed by in-gel tryptic digestion followed by mass spectrometry. The lower section shows the amino acid sequence for the first 93 residues of Cdt1, with the peptides recovered by mass spectrometry highlighted in boldface type. Asterisks indicate residues that were phosphorylated. B, GST-tagged Cdt1-(1–93) was purified from 293T cells after transfection and resolved by SDS-PAGE followed by Coomassie Blue staining. The left lane represents an untransfected sample used to identify nonspecific bands. The band corresponding to Cdt1 was excised and processed as described for panel A. The bottom section shows the amino acid sequence for the first 93 residues of Cdt1, with the peptides recovered by mass spectrometry highlighted in boldface type. Asterisks indicate residues that were phosphorylated.

Threonine 29 is important for Cdt1 interaction with Skp2—Based on the phosphopeptide mapping results, we constructed point mutants that had threonine 29 mutated to alanine, Cdt1(T29A), or serine 31 mutated to alanine, Cdt1(S31A). The Cy motif mutant Cdt1(ΔCy) was created by mutating residues 67–69, which correspond to RRL of the Cy motif, to AAA. This mutation has been shown to prevent the stable association of cyclins with Cdt1 in vitro and in vivo (16). We also constructed a mutant containing all three mutations Cdt1(T29A,S31A,ΔCy). These plasmids were transfected into cells in order to examine their interaction with Skp2 by immunoprecipitation. Wild-type Cdt1 and because Skp2 co-precipitated endogenous Skp2 (Fig. 3, lane 2). However, mutation of the Cy motif completely abolished the stable interaction between Cdt1 and Skp2 (Fig. 3, lane 5) as described previously (15, 16). Similarly, Cdt1(T29A) failed to co-precipitate Skp2 (Fig. 3, lane 3). In contrast, mutation of serine 31 to alanine had no effect on Skp2 association (Fig. 3, lane 4). The construct containing all three mutations also did not co-precipitate Skp2 as predicted. Therefore, we conclude that threonine 29 and the Cy motif are both necessary for Cdt1 interaction with Skp2. The position of threonine 29 is similar to that of previously described Cdk substrates that locate the Cy motif C-terminal to the Cdk phosphoacceptor sites (23). The distance between threonine 29 and the Cy motif is also greater than the minimal number of residues required for bipartite substrate recognition by cyclin A/Cdk2 (29).
and Cdt1($\Delta$Cy) to be resistant to proteasome-mediated degradation. Plasmids expressing WT or mutant Cdt1 were transfected into HeLa cells and harvested at G1 or S phase following a nocodazole block and release as done in Fig. 1. Cdt1($\Delta$93) was cotransfected to allow comparison between the point mutants and a stable form of Cdt1 within the same sample. Extracts were immunoblotted with cyclin A to confirm G1 and S phase of the cell cycle. As expected, Cdt1($\Delta$93) is stable in S phase (Fig. 4A). Surprisingly Cdt1(T29A) and Cdt1($\Delta$Cy) were degraded in S phase similar to WT Cdt1 and Cdt1(S31A). Degradation of Cdt1 was still proteasome-mediated, as treatment of cells with the proteasome inhibitor MG132 prior to harvesting stabilized Cdt1(T29A) and Cdt1($\Delta$Cy) during S phase (Fig. 4B). To rule out artifacts caused by pharmacological inhibition of the cell cycle, we also examined the stability of the different Cdt1 proteins in an unperturbed S phase by harvesting cells at various time points following release from nocodazole (Fig. 4C, lanes 1–8). Under these conditions, WT Cdt1 and Cdt1($\Delta$Cy) were still degraded as cells entered S phase (Fig. 4C, lanes 1–8), whereas Cdt1($\Delta$93) was stable (Fig. 4C, lanes 13–16). Therefore interaction with Skp2 is not required for the degradation of Cdt1 during S phase, suggesting the presence of Skp2-independent mechanisms for degrading Cdt1 during S phase.

Residues 1–32 of Cdt1 Are Required for S Phase-specific Degradation of Cdt1—Because Cdt1($\Delta$93) is stable in S phase, we hypothesized that there must be another signal within the first 93 amino acids that targets Cdt1 for degradation independent of Skp2 and cyclin binding. Therefore, we made truncation mutants of the N terminus of Cdt1 that deleted the first 19, 32, and 60 amino acids. When these mutants were analyzed during the cell cycle, Cdt1($\Delta$19) was degraded, whereas Cdt1($\Delta$32) and Cdt1($\Delta$60) were stable during S phase (Figs. 5A and 4C). The addition of MG132 during S phase also did not have an effect on the levels of Cdt1($\Delta$32) (Fig. 4B, lanes 7 and 8). Based on these results, we also tested Cdt1 with an internal deletion of residues 20–32, Cdt1($\Delta$20–32). However Cdt1($\Delta$20–32) was still degraded in S phase (Fig. 5B), suggesting that there may be two redundant signals in the N terminus of Cdt1 that signal Cdt1 degradation during S phase. Therefore, we conclude that residues 1–32 are necessary for degradation of Cdt1 during S phase.

Failure to Degrade Cdt1 Results in an Altered S Phase Progression—Having two forms of Cdt1 that were stable in S phase, we examined the significance of Cdt1 degradation during the cell cycle (Figs. 6 and 7). HeLa cells transfected with various Cdt1 plasmids were harvested at various time points following release from nocodazole. To analyze transfected cells by FACS, a plasmid expressing farnesylated GFP was cotransfected, and GFP-positive cells were counted. The cell cycle profile of cells expressing wild type Cdt1, Cdt1(T29A), or Cdt1($\Delta$Cy) were identical to control cells. However, the cell cycle progression of cells transfected with Cdt1($\Delta$32) or Cdt1($\Delta$93) was significantly different. After 6 h, Cdt1($\Delta$32)- and Cdt1($\Delta$93)-expressing cells had entered G1 and showed a FACS profile identical to the other Cdt1 constructs and control cells. However, at 12 and 18 h after release, cells transfected with Cdt1($\Delta$32) and Cdt1($\Delta$93) displayed predominantly 2n DNA characteristic of G1 phase, whereas the majority of cells transfected with wild type Cdt1, Cdt1(T29A), or Cdt1($\Delta$Cy) entered S phase and displayed greater than 2n DNA content. Even after 24 h when most of the control, wild type Cdt1-, Cdt1(T29A)-, and Cdt1($\Delta$Cy)-transfected cells had completed S phase, cells expressing Cdt1($\Delta$32) or Cdt1($\Delta$93) still had very few cells with greater than 2n DNA content (Fig. 7). The effect of stable Cdt1 expression appears to be a delayed entry into S phase rather than a complete cell cycle arrest, because the cells eventually display 4n DNA after 54 h (Fig. 9). The duration of S phase is also longer with Cdt1($\Delta$32) and Cdt1($\Delta$93). The earliest time point at which S phase cells are visible for control cells and cells expressing wild type Cdt1 is 12 h after release. At the 24- and 30-h time points the majority of these cells contain 4n DNA, indicating that it takes ~12–18 h to complete replication. However, cells expressing Cdt1($\Delta$32), which begin...
to enter S phase at the 24-h time point, are still in S phase 18 h later at the 42-h time point. Therefore, stabilizing Cdt1 also increases the length of S phase. The FACS profile of Cdt1(Δ93) was almost identical to Cdt1(Δ32), suggesting that the region of Cdt1 responsible for the effects seen with Cdt1(Δ93) is contained within the first 32 amino acids.

To determine whether the stable forms of Cdt1 were functioning as dominant negatives, we examined the loading of replication factors on chromatin after nocodazole release. The only essential function of Cdt1 known is the loading of the Mcm2-7 complex onto chromatin. After Mcm2-7 loading, replication can proceed in the absence of Cdt1. Total cell extract (TCE) and chromatin fractions were prepared from cells expressing wild type Cdt1, Cdt1(Δ32), or an empty expression plasmid at different time points during the cell cycle. Fig. 8 shows that both wild type Cdt1 and Cdt1(Δ32) associated with the chromatin fraction, with wild type Cdt1 being degraded at the 18 h time point corresponding to S phase. Mcm7 was associated with chromatin after release from nocodazole and remained on chromatin as cells entered S phase. Therefore, Mcm7 association with chromatin was unaffected by the expression of either wild type Cdt1 or Cdt1(Δ32), suggesting that Mcms were loaded at origins to the same extent as in control cells. To show that the loaded Mcm complexes were functional, we also examined the association of Cdc45 with chromatin. Cdc45 loading requires Mcm2-7 loading and therefore is an indicator of proper Cdt1 function. Both wild type Cdt1 and Cdt1(Δ32) show loading of Cdc45 at the 12-h time point similar to control cells, demonstrating that Cdt1 had successfully loaded Mcm2-7 complexes prior to S phase. Therefore, we rule out the possibility that stable forms of Cdt1 are functioning in a dominant negative fashion.

At 54 h after release, expression of wild type Cdt1 caused the appearance of cells with greater than 4n DNA due to re-replication as described previously (Fig. 9A) (10, 30). Although progression through S phase was unaffected by overexpressing Cdt1(T29A) and Cdt1(ΔCy), overexpression did increase the fraction of cells with greater than 4n DNA (Fig. 9B) from 30 to 50% and 60%, respectively, suggesting that Skp2 and Cdk phosphorylation may negatively regulate the ability of Cdt1 to cause re-replication. These effects are not due to stabilization of Cdt1 protein, as immunoblotting confirms that they are expressed at similar levels (Fig. 9C, lanes 1–3). In contrast, Cdt1(Δ32) and Cdt1(Δ93) expression resulted in cells containing primarily 4n DNA with a smaller fraction of cells with re-replicated DNA as compared with wild type Cdt1 (Fig. 9B). Therefore in addition to their effect on S phase progression, stable forms of Cdt1 show a reduced capacity to stimulate re-replication. However, the decrease in re-replication may be due to the problems with progression through S phase as discussed above.

**DISCUSSION**

Recently, it was demonstrated that Cdt1 interacts with Skp2 in a phosphorylation-dependent manner (14–16). Our data suggest that this event is mediated by cyclin/Cdk phosphorylation of threonine 29. Threonine 29 is phosphorylated by cyclin/Cdk complexes in vitro and was found to be phosphorylated in vivo. Mutation of either threonine 29 or the Cy motif abolishes binding of Cdt1 to Skp2 in vivo (Fig. 3). Therefore, binding to...
cyclin is insufficient to promote association with Skp2, but modification of threonine 29 must also occur, most likely by being phosphorylated by cyclin/Cdk5. Similarly, Cdt1 that cannot associate with cyclins is unable to bind Skp2 even with a phosphorylatable threonine 29. If another kinase were responsible for phosphorylating threonine 29, then a Cy motif mutant would be expected to associate with Skp2. Hence, cyclin binding and phosphorylation of threonine 29 are probably interrelated events. The importance of phospho-threonine is consistent with previously described substrates of SCFSkp2, all of which thus far require a phosphorylated threonine. We cannot rule out the possibility that cyclins/Cdk5 recruit another kinase that then modifies threonine 29. However threonine 29 is contained within a consensus Cdk phosphoacceptor site so that if another kinase is involved, it would be another Cdk or a mitogen-activated protein kinase, the only kinases known to preferentially phosphorylate (S/T)P sequences. In addition, the role of a Cy motif in promoting the phosphorylation of a Cdk substrate site by a Cdk is reminiscent of our earlier studies on Cy motifs and Cdk phosphorylation sites in another replication factor, Cdc6 (29). In that example, a Cy motif situated at least 12–16 residues C-terminal to the Cdk substrate site significantly decreased the Km of a substrate for phosphorylation by both cyclin E/Cdk2 and cyclin A/Cdk2. Thus we suggest that a Cy motif-directed interaction with cyclin A/Cdk2 promotes the phosphorylation of threonine 29 of Cdt1 by cyclin A/Cdk2. This phosphorylation is necessary for Cdt1 to interact with Skp2.

Interestingly, mutants of Cdt1 that do not interact with Skp2 or cyclins are degraded during S phase similar to wild type Cdt1 protein (Figs. 4 and 5). This does not rule out the possibility that Skp2 is involved in degrading Cdt1, but it demonstrates that there are Skp2-independent mechanisms that degrade Cdt1 during S phase. Cdk phosphorylation may still be required for indirectly degrading Cdt1, but the stable association of Cdt1 with cyclins is not necessary. One mechanism involves the N-terminal 32 residues of Cdt1, as deletion of this region stabilizes Cdt1 during S phase. Previously, stabilization of Cdt1 was reported following Skp2 RNA interference, which implies that Skp2 is rate-limiting for Cdt1 degradation (14). However silencing of Skp2 has been shown to stabilize multiple targets including p27 (20, 21, 31), resulting in G1 arrest at
findings. In recent reports in other organisms are also consistent with our Because we used Cdt1 point mutants, cell cycle effects caused precisely the time during the cell cycle when Cdt1 is stable. degradation. Phosphorylation of Cdt1 has been shown to affect its ability to bind DNA (16). Other pre-RC components such as ORC and Cdc6 are also negatively regulated by CDK phosphorylation independent of proteolysis (32–35). Therefore, it is conceivable that the interaction of Cdt1 with ORC or with Cdc6 or its function in loading Mcm2-7 proteins is negatively regulated by phosphorylation on threonine 29.

Consistent with the hypothesis that Cdt1 proteolysis is mediated through Skp2-independent mechanisms, the cell cycle progression of cells expressing cyclin and Skp2 binding mutants resemble that of wild type Cdt1 rather than Cdt1(Δ93). Wild type Cdt1, Cdt1(ΔCy), and Cdt1(T29A) progress through S phase with the same kinetics as control cells (Figs. 6 and 7). However, cells expressing Cdt1(Δ93) show a significantly delayed entry into and completion of S phase, suggesting that failure to degrade Cdt1 prevents the efficient progression through S phase. Although neither the Cy motif nor Skp2 binding can explain the requirement of the N terminus for Cdt1 proteolysis, deletion of the first 32 residues of Cdt1 was sufficient to prevent degradation of Cdt1 during S phase. Cells expressing Cdt1(Δ32) also displayed the same cell cycle phenotype as Cdt1(Δ93), supporting the notion that the effects seen with Cdt1(Δ93) are due to its stabilization. It is currently unclear why failure to degrade Cdt1 prevents efficient S phase progression. One possibility is that the stable forms of Cdt1 act as dominant negatives that interfere with the normal function of Cdt1. The only known function of Cdt1 act as dominant negatives that interfere with the normal function of Cdt1.

According to our results, degradation of mammalian Cdt1 in S phase after entering G2. These findings are consistent with previous data showing that overexpression of Cdt1 stimulates re-replication and activates checkpoint pathways, preventing entry into mitosis (10–12). Skp2 and cyclin binding mutants of Cdt1 enhance the re-replication seen with wild type Cdt1 inprogression of Cdc45 to chromatin do not require the presence of Cdt1. Because both Mcm and Cdc45 loading were unaffected in cells expressing a stable form of Cdt1, we conclude that the abnormal S phase progression is not due to inhibition of wild type Cdt1 function. Furthermore, stable forms of Cdt1 stimulate re-replication, which also argues against a dominant negative mechanism. We speculate that the persistence of Cdt1 causes re-loading during early S phase. Re-loading may activate checkpoints that monitor inappropriate pre-RC formation and respond by arresting or slowing down the cell cycle. Alternatively, degradation of Cdt1 may be required for efficient origin firing by a previously uncharacterized mechanism. Lastly, the N terminus of Cdt1 may encode a separate function that promotes DNA synthesis.

Although cells overexpressing wild type Cdt1 progressed through S phase similar to controls, re-replication became apparent after entering G2. These findings are consistent with previous data showing that overexpression of Cdt1 stimulates re-replication and activates checkpoint pathways, preventing entry into mitosis (10–12). Skp2 and cyclin binding mutants of Cdt1 enhance the re-replication seen with wild type Cdt1 independent of proteolysis. Similar findings were reported with Drosophila Cdt1, where mutation of Cdk phosphorylation sites resulted in increased re-replication when overexpressed (30). Recently it was shown that inhibition of Cdk activity during mitosis was sufficient to cause reloading of Cdt1 on chromatin (25). Therefore, it is possible that Cdk phosphorylation is important during a window in G2/M phase for preventing Cdt1 reloading and origin refiring, whereas geminin is the main inhibitor of Cdt1 function during the remainder of the cell cycle. Interestingly, mutations that stabilize Cdt1 do not produce the overt re-replication seen with wild type Cdt1. This observation may be related to the aberrant S phase, which preceded entry into G2. Preventing efficient S phase progression by stabilizing Cdt1 may likewise impede re-replication. An alternative hypothesis is that origins need to cleared of geminin-bound Cdt1 before they can reinitiate replication. Xenopus geminin has been shown to accumulate on chromatin after Cdt1 loading to inhibit Cdt1 function and is released only after Cdt1 is degraded (22, 36). Therefore without Cdt1 turnover,
geminin remains at origins and prevents re-replication. It will be interesting to generate stable Cdt1 mutants that fail to interact with geminin to test these hypotheses.

In summary, we support previous findings demonstrating the importance of the N terminus of Cdt1 for its degradation during S phase. However, although the N terminus of Cdt1 is required for both cyclin and Skp2 binding, we find that neither is required for degrading Cdt1 during S phase. Therefore, Cdt1 proteolysis during S phase is mediated via Skp2-independent pathways. In the process of localizing the degradation signal to the first 32 amino acids of Cdt1, we discovered that overexpression of stable forms of Cdt1 results in a delayed entry into and progression through S phase. In contrast, Cdt1 mutants that fail to interact with Skp2 and cyclins stimulate the re-replication seen when overexpressing Cdt1, even though they do not stabilize Cdt1 in S phase. This latter finding is particularly important, because it clearly indicates that phosphorylation by Cdk2 and interaction with Skp2 can have a negative effect on Cdt1 function independent of targeting it for degradation.

These findings reconcile some conflicting findings in the literature and provide novel insight into the multiple controls in place for regulating Cdt1. In mammalian cells, Cdt1 overexpression has been shown to have oncogenic potential (37), suggesting that deregulating Cdt1 could be contributory to the development of cancer. Therefore, understanding the mechanisms involved in regulating Cdt1 might reveal mechanisms by which cancer cells escape these controls, leading to re-replication and genomic instability.

REFERENCES
1. Blow, J. J., and Hodgson, B. (2002) Trends Cell Biol. 12, 72–78
2. Nishitani, H., and Lygerou, Z. (2002) Genes Cells 7, 523–534
3. Bell, S. P., and Dutta, A. (2002) Annu. Rev. Biochem. 71, 353–374
4. Brock, D., Bartlett, R., Crawford, K., and Nurse, P. (1991) Nature 349, 388–393
5. Dahmann, C., Diffler, J. F., and Nasmyth, K. A. (1995) Curr. Biol. 5, 1257–1269
6. Itzhaki, J. E., Gilbert, C. S., and Porter, A. C. (1997) Nat. Genet. 15, 258–265
7. Tada, S., Li, A., Maiorano, D., Mechali, M., and Blow, J. J. (2001) Nat. Cell Biol. 3, 107–113
8. Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000) Science 290, 2309–2312
9. Lee, C., Hong, B., Choi, J. M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., and Cho, Y. (2004) Nature 430, 913–917
10. Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003) Mol. Cell 11, 997–1008
11. Zhu, W., Chen, Y., and Dutta, A. (2004) Mol. Cell. Biol. 24, 7140–7150
12. Mellitzer, M., Balabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., Lukas, J., and Helin, K. (2004) J. Cell Biol. 165, 473–482
13. Nishitani, H., Taraviras, S., Lygerou, Z., and Nishimoto, T. (2001) J. Biol. Chem. 276, 44905–44911
14. Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003) J. Biol. Chem. 278, 30854–30858
15. Liu, E., Li, X., Yan, F., Zhao, Q., and Wu, X. (2004) J. Biol. Chem. 279, 17283–17288
16. Sugimoto, N., Tatsuki, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H., and Fujita, M. (2004) J. Biol. Chem. 279, 19691–19697
17. Nishitani, H., Lygerou, Z., and Nishimoto, T. (2004) J. Biol. Chem. 279, 30807–30816
18. Nakayama, K., Nagahama, H., Minamishima, Y. A., Miyake, S., Ishida, N., Hatakaiyama, S., Kitagawa, M., Iemura, S., Natsume, T., and Nakayama, K. I. (2004) Dev. Cell 6, 661–672
19. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. (1995) Cell 82, 915–925
20. Kossatz, U., Dietrich, N., Zender, L., Buer, J., Manns, M. P., and Malek, N. P. (2004) Genes Dev. 18, 2602–2607
21. Carrano, A. C., Ethian, E., Hershko, A., and Pagnino, M. (1999) Nat. Cell Biol. 1, 193–199
22. Arias, E. E., and Walter, J. C. (2005) Genes Dev. 19, 114–126
23. Wohlschlegel, J. A., Dwyer, B. T., Takeda, D. Y., and Dutta, A. (2001) Mol. Cell 21, 4868–4874
24. Delmolino, L. M., Saha, P., and Dutta, A. (2001) J. Biol. Chem. 276, 26947–26954
25. Ballabeni, A., Melixetian, M., Zamponi, R., Masiero, L., Marinoni, F., and Helin, K. (2004) EMBO J. 23, 3122–3132
26. Pacek, M., and Walter, J. C. (2004) EMBO J. 23, 3667–3676
27. Li, A., and Blow, J. J. (2005) EMBO J. 24, 395–404
28. Perrenthuth, A., Li, A., Brito-Martins, M., and Blow, J. J. (2005) Nucleic Acids Res. 33, 316–324
29. Takeda, D. Y., Wohlschlegel, J. A., and Dutta, A. (2001) J. Biol. Chem. 276, 1993–1997
30. Theimer, M., May, N. R., Aggarwal, B. D., Kwock, G., and Calvi, B. R. (2004) Development 131, 4807–4818
31. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senftlen, M., Muller, U., and Krek, W. (1999) Nat. Cell Biol. 1, 207–214
32. Li, C. J., Vassilev, A., and DePamphilis, M. L. (2004) Mol. Cell. Biol. 24, 5875–5886
33. Wilmes, G. M., Archambault, V., Austin, R. J., Jacobson, M. D., Bell, S. P., and Cross, F. R. (2004) Genes Dev. 18, 981–991
34. Nguyen, V. Q., Co, C., and Li, J. J. (2004) Nature 411, 1068–1073
35. Alexander, M. G., and Hamlin, J. L. (2004) Mol. Cell 24, 1614–1627
36. Maiorano, D., Rul, W., and Mechali, M. (2004) Exp. Cell Res. 295, 138–149
37. Arntzen, C., Falcon, P., See, J., Moen, E., Studis, J. M., Premont, D. H., and Choi, K. (2002) Oncogene 21, 1150–1158