Eukaryotic cells tightly control DNA replication so that replication origins fire only once during S phase within the same cell cycle. Cell cycle-regulated degradation of the replication licensing factor Cdt1 plays important roles in preventing more than one round of DNA replication per cell cycle. We have previously shown that the SCFSkp2-mediated ubiquitination pathway plays an important role in Cdt1 degradation. In this study, we demonstrate that human Cdt1 is a substrate of Cdk2 and Cdk4 both in vivo and in vitro. Overexpression of cyclin-dependent kinase inhibitors such as p21 and p27 dramatically suppresses the phosphorylation of Cdt1, disrupts the interaction of Cdt1 with the F-box protein Skp2, and blocks the degradation of Cdt1. Further analysis reveals that Cdt1 interacts with cyclin/cyclin-dependent kinase (Cdk) complexes through a cyclin/Cdk binding consensus site, located at the N terminus of Cdt1. A Cdt1 mutant carrying four amino acid substitutions at the Cdk binding site dramatically reduces associations with cyclin/Cdk complexes. This mutant is not phosphorylated, fails to bind Skp2 and is more stable than wild-type Cdt1. These data suggest that cyclin/Cdk-mediated Cdt1 phosphorylation is required for the association of Cdt1 with the SCFSkp2 ubiquitin ligase and thus is important for the cell cycle-dependent degradation of Cdt1 in mammalian cells.

The replication licensing factor Cdt1 is an essential component of the pre-replication complex and is required for loading MCM proteins onto chromatin (1–6). Cdt1 was originally isolated in fission yeast and is conserved in different organisms (1). In fission yeast, Cdt1 cooperates with another essential replication initiation regulator Cdc18 (Cdc6 homolog in Schizosaccharomyces pombe) to initiate DNA replication (4). Immunodepletion of Cdt1 in Xenopus egg extracts prevents DNA replication in an in vitro DNA replication system (7). Microinjection of a Cdt1 antibody in human cells prevents DNA replication initiation (6).

The protein levels of Cdt1 in fission yeast and in mammalian cells are tightly controlled (4, 8). Human Cdt1 protein is present in G1 phase of the cell cycle and is degraded as cells enter S phase (9). Cdt1 in higher eukaryotes binds geminin, a DNA replication inhibitor that is present in S and G2, and is inactivated by geminin (5). The tight control of Cdt1 protein level during cell cycle, together with the observation that overexpression of Cdt1 alone or with Cdc6/Cdc18 induces DNA re-replication (9, 10), suggests that the cell cycle-dependent inactivation of Cdt1 is essential for preventing inappropriate DNA replication initiation.

CdkS play major roles in regulating cell cycle progression (11, 12). In mammalian cells, cyclin-dependent kinase complexes operate the passage through the G1 phase of the cell cycle. Subsequently, the CyclinE/Cdk2 complex is formed and promotes the G1 to S transition, and the CyclinA/Cdk2 complex regulates the S phase progression. In mammalian cells, the activity of CdkS can be inhibited by the interaction of cyclin/Cdk complexes with Cdk inhibitors, such as p21 and p27 (12). It was demonstrated that the elevated Cdk activity prevents re-initiation of DNA replication in S and G2 from origins that have fired (13, 14). In yeast, Cdc6 (Cdc18) are phosphorylated by CdkS and are subsequently targeted for ubiquitin-dependent proteolysis (15–18). In mammalian cells, Cdc6 is also a substrate of CdkS and the phosphorylation regulates its subcellular localization (19, 20). However, the post-translational modification of Cdt1 remains to be investigated.

We previously showed that cell cycle regulated Cdt1 degradation in mammalian cells is mediated through the SCFSkp2 ubiquitination pathway (21). Specifically, Cdt1 interacts with the F-box protein Skp2 in a phosphorylation dependent manner and the SCFSkp2 ubiquitin ligase complex targets Cdt1 for degradation. In the presence of proteasome inhibitor MG132, the phosphorylated Cdt1 species are accumulated. These studies suggest that phosphorylation of Cdt1 may be important for the controlled degradation of Cdt1 when cells enter S phase. Here, we show that cyclin/Cdk complexes interact with and phosphorylate Cdt1 and these phosphorylation modifications are required for the association of Cdt1 with Skp2 and thereby important for Cdt1 degradation.

EXPERIMENTAL PROCEDURES

Plasmids—Myc-tagged full-length human Cdt1 and various Cdt1 deletion mutants were described previously (21). The mutant carrying a deletion of residues 62 to 72 was generated by ligating PCR products encoding residues 1–62 (AgaI-Nhel) and 72–546 (Nhel-XhoI) with the vector pCMMA3 (22) that carries the sequence encoding the Myc epitope after AgaI and XhoI digestion. The NAAIRS mutant was generated by ligating the PCR product encoding the residues 1–64 followed by the amino acids NAAIRS included in the 3′-primer (ATCGATCGG-ATGGCGGCGTTG GCTGGCCTGGTCGCGTCCGGGG; underline,enda
Cdk Complexes Phosphorylate Cdt1.

**A** (17284)

**Human Cdt1 Is a Substrate of Cdk2 in Vivo**—In yeast, Cdc6 and Cdc18 are Cdk substrates and Cdk-mediated phosphorylation triggers Cdc6/Cdc18 degradation (15–18). To examine whether Cdk2 phoshozylates Cdt1 in mammalian cells, we overexpressed Cdk2 and Cdk4 with or without co-expression of cyclinD1, cyclinE and cyclinA in 293T cells. Transient transfection of Cdk2 and Cdk4 induced phosphorylation of endogenous Cdt1, as revealed by increased density and a more dramatic shift of the slower migrating Cdt1 bands (Fig. 1A). Co-transfection of cyclinA, cyclinE, or cyclinD1 along with Cdk2 and Cdk4 further enhanced Cdt1 phosphorylation. A phosphatase treatment of cell lysate abolished the slower migrating bands, confirming that the shift of Cdt1 bands was caused by Cdk-mediated phosphorylation.

To further investigate whether Cdt1 is phosphorylated by Cdk2, we overexpressed Cdk inhibitors p21 and p27 and examined Cdt1 phosphorylation. 293T cells were transfected with p21, p27, or the vector and lysed before and after MG132 treatment. In the vector-transfected cells, endogenous Cdt1 exhibited a doublet even without MG132 treatment (Fig. 1B, second lane). In the presence of MG132, the top band can be abolished by phosphatase treatment (Fig. 1B, first lane). In the presence of MG132, the Cdt1 species with even slower mobility accumulated. A phosphatase treatment indicated that the slower migrating species of Cdt1 was the phosphorylated form of Cdt1. When p21 or p27 was expressed, in the absence of MG132, the top band of the doublet present in the vector-transfected cells is diminished. In the presence of MG132, the expression of p21 or p27 dramatically decreased the amount of slower migrating species of Cdt1, and the lower band was accumulated. These results demonstrate that overexpression of p21 and p27 inhibits Cdt1 phos-
phosphorylation and suggest that Cdk activities are required for the phosphorylation of Cdt1 in mammalian cells.

Cdk2 and Cdk4 Phosphorylate Cdt1 in Vitro—To more directly test whether Cdt1 is a substrate of Cdkks, we performed an in vitro kinase assay. 293T cells were transfected with FLAG-Cdk2, FLAG-Cdk4, or vector. Cdk2 and Cdk4 were purified by anti-FLAG immunoprecipitation and eluted by the FLAG-epitope. The Cdk2/Cdk4-associated full-length Cdt1 was used as a substrate. As shown in Fig. 1C, Cdt1 was phosphorylated by Cdk2 and Cdk4 in vitro, and the phosphorylation was inhibited in the presence of Cdk inhibitor roscovitine.

The C-terminal 168 Residues Are Important for Cdk-mediated Phosphorylation of Cdt1—To examine the regions of Cdt1 that can be phosphorylated by Cdkks in vivo, we co-transfected 293T cells with cyclinA/Cdk2 or cyclinE/Cdk2 and the Myc-tagged full-length Cdt1 or Cdt1 C-terminal deletion mutants. Overexpression of cyclinA/Cdk2 or cyclinE/Cdk2 retarded the migration of the full-length Cdt1 (1–546) and the fragment 1–417 but not the fragments 1–378 and 1–285 (Fig. 1D). λ-Phosphatase treatment abolished the migration shift (data not shown), confirming that overexpression of cyclinA/Cdk2 and cyclinE/Cdk2 induces phosphorylation of the full-length Cdt1 and fragment 1–417. These results suggest that the C-terminal 168 residues that are absent in the Cdt1 fragment 1–378 are important for the Cdk-mediated phosphorylation of Cdt1, and the region between residues 378 and 417 may contain major Cdk phosphorylation sites or other elements that are required for Cdk-mediated Cdt1 phosphorylation.

Inhibition of Cdk Activities Suppresses the Association of Cdt1 with Skp2 and Blocks the Degradation of Cdt1—We showed that the interaction of Cdt1 and Skp2 is phosphorylation-dependent. To test whether Cdk-mediated phosphorylation of Cdt1 is important for this association, we transfected 293T cells with Cdk inhibitors, p21 and p27. The interaction of Cdt1 and Skp2 was examined by co-immunoprecipitation of Skp2 with Cdt1 (Fig. 2A). Overexpression of p21 and p27 dramatically reduced the association of Cdt1 with Skp2. This suggests that Cdk activities are required for Cdt1 to bind Skp2.

The SCFSkp2-dependent ubiquitination pathway plays an important role for Cdt1 degradation (21). As inhibition of Cdk kinase activities suppresses the association of Cdt1 with Skp2, we tested whether Cdk activities are important for Cdt1 degradation. Overexpression of p21 and p27 led to accumulation of endogenous Cdt1 (Fig. 2B). The stability of Cdt1 was measured. As shown in Fig. 2C, Cdt1 was significantly more stable when p21 and p27 were overexpressed. Taken together, these data suggest that the phosphorylation of Cdt1 by cyclin/Cdk complexes triggers the association of Cdt1 with Skp2, thereby inducing Cdt1 degradation.

Cdt1 Interacts with Cyclin/Cdk2 and Cyclin/Cdk4 Complexes through a Cyclin Binding Consensus Site at the NTerminus of Cdt1—Cdk complexes with cyclinA or cyclinE interacted with Cdt1 (Fig. 1B). The SCFSkp2-dependent ubiquitination pathway plays an important role for Cdt1 degradation (21). As inhibition of Cdk kinase activities suppresses the association of Cdt1 with Skp2, we tested whether Cdk activities are important for Cdt1 degradation. Overexpression of p21 and p27 led to accumulation of endogenous Cdt1 (Fig. 2B). The stability of Cdt1 was measured. As shown in Fig. 2C, Cdt1 was significantly more stable when p21 and p27 were overexpressed. Taken together, these data suggest that the phosphorylation of Cdt1 by cyclin/Cdk complexes triggers the association of Cdt1 with Skp2, thereby inducing Cdt1 degradation.

To search for regions in Cdt1 that mediate the cyclin/Cdkks binding, a series of Cdt1 deletion mutants were co-expressed with HA-cyclinA/FLAG-Cdk2, HA-cyclinE/FLAG-Cdk2, or cyclinD1/FLAG-Cdk4 in 293T cells. Deletion of the N-terminal 52 residues significantly reduced Cdt1 binding with cyclinE/Cdk2 and cyclinA/Cdk2 complexes, and Cdk4 when they were overexpressed in 293T cells (Fig. 3, B–D; vector versus full-length Cdt1 (FL)).
Cdk Complexes Phosphorylate Cdt1

The Interaction of Cdt1 with Cdk Complexes Is Important for Cdt1 Phosphorylation—To test whether the interactions of Cdt1 and Cdk complexes are important for the phosphorylation of Cdt1, we generated stable 293 cell lines that express the Myc-tagged wild-type Cdt1 and the Myc-NAAIRS Cdt1 mutant by retroviral infection. Myc-tagged Cdt1 migrates more slowly than endogenous Cdt1, thus the anti-Cdt1 Western blot analysis shows the tagged and the endogenous Cdt1 species as two separate bands. As illustrated in Fig. 4A, treatment with MG132 induced a migration shift of Myc-Cdt1-WT and endogenous Cdt1, which can be abolished by phosphatase treatment. However, no obvious phosphorylation of Myc-Cdt1 NAAIRS mutant was observed after MG132 treatment, although at the same time the endogenous Cdt1 was phosphorylated. These data demonstrate that the Cdt1 NAAIRS mutant defective in Cdk binding is not phosphorylated efficiently in vivo and suggest that the association of Cdt1 with Cdk complexes is important for Cdt1 phosphorylation.

The Cdt1 Mutant Defective in Cdk Association Does Not Bind to Skp2 and Is More Stable than Wild-type Cdt1—As the Cdt1 NAAIRS mutant is defective in phosphorylation, we examined whether this mutant was able to bind Skp2. Immunoprecipitation of Skp2 from the 293 stable cell lines that expressed Myc-Cdt1-WT or Myc-NAAIRS Cdt1 showed that endogenous Cdt1 and Myc-Cdt1-WT, but not the Myc-Cdt1-NAAIRS mutant, interacted with Skp2 (Fig. 4B, left). Anti-Myc immunoprecipitation also demonstrated that Myc-Cdt1-WT, but not the Myc-Cdt1-NAAIRS mutant, associated with endogenous Skp2 (Fig. 4B, middle).

The Cdt1 NAAIRS mutant is more stable than wild-type Cdt1. 293 cell lines that stably express Myc-Cdt1-WT or Myc-Cdt1-NAAIRS were treated with cycloheximide (CHX) and subsequently lysed at different time points. Anti-Myc immunoblotting showed that Myc-Cdt1-NAAIRS degraded more slowly than Myc-Cdt1-WT (Fig. 4C, top). Consistently, anti-Cdt1 Western blot indicated that the Myc-Cdt1-NAAIRS mutant, but not Myc-Cdt1-WT, was more stable than endogenous Cdt1 (Fig. 4C, bottom). These results suggest that Cdt1 phosphorylation is required for Cdt1 to associate with Skp2 and therefore important for the degradation of Cdt1.

DISCUSSION

The Cdt1 protein levels fluctuate during the cell cycle and proteolysis appears to be a primary mechanism for the periodic accumulation of Cdt1 (8). MG132 treatment stabilizes Cdt1 and leads to the accumulation of the phosphorylated species of Cdt1, suggesting that phosphorylation of Cdt1 may play an important role for targeting Cdt1 for degradation (21). Here we provide evidence that Cdt1 is a substrate of Cdk2 and Cdk4 both in vivo and in vitro. Cdt1 interacts with multiple cyclin/Cdk complexes, including cyclinE/Cdk2, cyclinA/Cdk2, and Cdk4 complexes. Inhibition of Cdk activities suppresses Cdt1 phosphorylation and leads to the stabilization of Cdt1. Furthermore, a NAAIRS Cdt1 mutant defective in Cdk binding is no longer phosphorylated and exhibits greater stability than wild-type Cdt1. These results suggest that cyclin/Cdk complexes phosphorylate Cdt1 and these phosphorylation events are important for Cdt1 degradation. The contribution of each individual cyclin/Cdk complex to this process is currently under investigation.

We have shown that the degradation of Cdt1 is mediated at least in part by the SCFSkp2-dependent ubiquitination pathway (21). The interaction of Cdt1 and Skp2 is phosphorylation-dependent. Our study demonstrates that the association of Cdt1 with Skp2 can be abolished by the inhibition of Cdt1 phosphorylation when p21 or p27 is overexpressed or when a Cdk binding mutant (NAAIRS) is used. These results are consistent with a model that Cdt1 is phosphorylated by Cdk complexes as cells pass through G1 and enter S phase. Consequently, the phosphorylated Cdt1 associates with the SCFSkp2 complex through Skp2 and is degraded through ubiquitination pathway.
Cdt1 is a replication licensing factor that is required for the initiation of DNA replication (4–6). In addition, the levels of Cdt1 need to be tightly regulated to prevent origin re-firing, as overexpression of Cdt1 in p53-deficient cells leads to DNA re-replication (10). Under the phosphorylation control by Cdk complexes, Cdt1 becomes a substrate of SCFSkp2 and degrades when cells enter S phase. This regulation allows Cdt1 to accumulate only in G1 and thus prevents the formation of pre-replication complex after DNA replication starts, which is important for the prevention of DNA re-replication. Therefore, Cdk-mediated and cell cycle-regulated Cdt1 degradation may be an important mechanism ensuring strict control of DNA replication and the maintenance of genome stability.

**Acknowledgments**—We thank Ernest Beutler and Bernard Babior for support. We thank Thomas Bray for comments and criticism and for reading the manuscript and members of the Wu laboratory for discussions.

**REFERENCES**

1. Bell, S. P., and Dutta, A. (2002) *Annu. Rev. Biochem.* **71**, 333–374
2. Blow, J. J., and Hodgson, B. (2002) *Trends Cell Biol.* **12**, 72–78
3. Difley, J. F. (2001) *Curr. Biol.* **11**, R367–R370
4. Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000) *Nature* **404**, 625–628
5. Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000) *Science* **290**, 2309–2312
6. Rialland, M., Sola, F., and Santocanale, C. (2002) *J. Cell Sci.* **115**, 1435–1440
7. Maiorano, D., Moreau, J., and Mechali, M. (2000) *Nature* **404**, 622–625
8. Nishitani, H., Taraviras, S., Lygerou, Z., and Nishimoto, T. (2001) *J. Biol. Chem.* **276**, 44905–44911

![Diagram A](image1.png)

**Fig. 4. Defects of the Cdk binding mutant of Cdt1 (Cdt1-NAAIRS).** A, the Cdt1-NAAIRS mutant is defective in phosphorylation. Stable 293 cell lines that express Myc-tagged wild-type Cdt1 or Cdt1 NAAIRS mutant were generated by retroviral infection. Cells were lysed before and after MG132 treatment (10 μM, 3 h). Anti-Cdt1 Western blot analysis was performed using cell lysates treated with or without λ-phosphatase (pptase). IB, immunoblotting. B, the Cdt1-NAAIRS mutant does not bind Skp2. Immunoprecipitation was performed using cell lysates purified from stable 293 cell lines that express Myc-tagged wild-type Cdt1 or Cdt1 NAAIRS mutant or that was infected by the empty retroviral vector. Endogenous Skp2 was immunoprecipitated with anti-Skp2 polyclonal antibody, and the immunoprecipitates were immunoblotted with affinity-purified anti-Cdt1 polyclonal antibody (left). Anti-Myc immunoprecipitation was performed by using 9E10 antibody. The association of Skp2 with Myc-Cdt1-WT and Myc-Cdt1-NAAIRS was demonstrated by anti-Skp2 (monoclonal antibody, Zymed Laboratories Inc.) Western blot (middle). The expression of Myc-Cdt1-WT and Myc-Cdt1-NAAIRS and endogenous Cdt1 in the 293 stable cell lines were examined by anti-Cdt1 Western blot (right). C, the Cdt1-NAAIRS mutant is more stable than wildtype Cdt1. 293 cell lines that express Myc-tagged wild-type Cdt1 or Myc-Cdt1 NAAIRS mutant were treated with CHX (100 μM) and lysed at the indicated time after CHX treatment for Western blot analysis. Anti-Myc (9E10) and anti-Cdt1 Western blot analysis was performed. Actin was used as a loading control. Quantitative analysis was performed by the measurement of Cdt1 band intensities normalized by the band intensities of actin at each time point. The percentage of the remaining of Cdt1 after CHX treatment was plotted over time (hour).
9. Yanow, S. K., Lygerou, Z., and Nurse, P. (2001) Embo J. 20, 4648–4656
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. Nasmyth, K. (1996) Science 274, 1643–1645
12. Hengstschlager, M., Braun, K., Soucek, T., Miloloza, A., and Hengstschlager-Ottad, K. (1999) Mutat. Res. 436, 1–9
13. Nishitani, H., and Nurse, P. (1995) Cell 83, 397–405
14. Dahmann, C., Diffley, J. F., and Nasmyth, K. A. (1995) Curr. Biol. 5, 1257–1260
15. Baum, B., Nishitani, H., Yanow, S., and Nurse, P. (1998) Embo J. 17, 5689–5698
16. Konimami, K., and Toda, T. (1997) Genes Dev. 11, 1548–1560
17. Jallepalli, P. V., Brown, G. W., Muzi-Falconi, M., Tien, D., and Kelly, T. J. (1997) Genes Dev. 11, 2767–2779
18. Drury, L. S., Perkins, G., and Diffley, J. F. (1997) Embo J. 16, 5966–5976
19. Jiang, W., Wells, N. J., and Hunter, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6193–6198
20. Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J., and Helin, K. (1999) Embo J. 18, 396–410
21. Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003) J. Biol. Chem. 278, 30854–30863
22. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D. M. (1997) Cell 88, 265–275
23. Wu, X., Ranganathan, V., Weisman, D. S., Heine, W. F., Ciccone, D. N., O’Neill, T. B., Crick, K. E., Pierce, K. A., Lane, W. S., Rathbun, G., Livingston, D. M., and Weaver, D. T. (2000) Nature 405, 477–482
24. Sun, P., Dong, P., Dai, K., Hannon, G. J., and Beach, D. (1998) Science 282, 2270–2272
25. Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M., and Kaelin, W. G., Jr. (1996) Mol. Cell. Biol. 16, 6623–6633
26. Schulman, B. A., Lindstrom, D. L., and Harlow, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10453–10458
27. Wilson, I. A., Haft, D. H., Getzoff, E. D., Tainer, J. A., Lerner, R. A., and Brenner, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5255–5259
Cyclin-dependent Kinases Phosphorylate Human Cdt1 and Induce Its Degradation
Enbo Liu, Xianghong Li, Feng Yan, Qiping Zhao and Xiaohua Wu

J. Biol. Chem. 2004, 279:17283-17288.
doi: 10.1074/jbc.C300549200 originally published online March 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.C300549200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 16 of which can be accessed free at
http://www.jbc.org/content/279/17/17283.full.html#ref-list-1