Human CDT1 Associates with CDC7 and Recruits CDC45 to Chromatin during S Phase*5

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The initiation of DNA replication is a tightly controlled process that involves the formation of distinct complexes at origins of DNA replication at specific periods of the cell cycle. Pre-replicative complexes are formed during telophase and early G1. They rearrange at the start of S phase to form pre-initiation complexes, which are a prerequisite for DNA replication. The CDT1 protein is required for the formation of the pre-replicative complexes. Here we show that human CDT1 associates with the CDC7 kinase and recruits CDC45 to chromatin. Moreover, we show that the amount of CDT1 bound to chromatin is regulated by CDC7. We propose a model in which chromatin-bound CDT1 is first stabilized and subsequently displaced by CDC7 activity, thereby ensuring the timely execution of DNA replication.

The initiation of DNA replication is a tightly regulated process involving the orderly recruitment of specific proteins to the origins of DNA replication. In current models for DNA replication, the origins are activated (fired) by the CDC7 kinase at different times during S phase (1, 2). To license the origins for DNA replication multiprotein complexes, called pre-replicative complexes (pre-RCs),5 have to be formed. ORC proteins bind to the origins of DNA replication throughout the cell cycle, and the binding of CDC6 and CDT1 and the subsequent recruitment of the hexameric complex MCM2–7 lead to pre-RC formation in late mitosis (telophase) and early G1 (3, 4). CDC6 and CDT1 are both required for pre-RC formation (5–9). By recruiting other replication factors the pre-RCs rearrange during S phase and form pre-initiation complexes (pre-ICs). Pre-ICs form only after the activation of CDK2 and CDC7 at the G1/S transition (10–12). The CDC45 protein is a replication factor recruited to chromatin in a CDC7-dependent manner and is required for pre-IC formation and activation of the replication origins (13–19). DBF4 binding to CDC7 activates the kinase, and this has been shown to regulate the firing of each single replication origin in S. cerevisiae, suggesting that the kinase is activated at different times during S phase (1, 2, 20–24). Following CDC45 association, unwinding of double-stranded DNA precedes the recruitment of the single strand binding protein RPA and onset of DNA synthesis (25). The CDT1-interacting protein, Geminin, together with CDK activity, prevents the firing of the same origin of replication during S-G2 phases, assuring that each portion of the DNA is replicated once and only once per cell cycle (26–34).

The function of pre-RC components during S phase has not been clearly elucidated. It is known that the pre-RCs are disassembled as cells exit S phase, and only ORC proteins remain on the site to establish a complex, the so-called post-replicative complex (post-RC), representing a landing platform for pre-RC formation at the following G1/S transition (3). MCM proteins have been found to have in vitro helicase activity and have been hypothesized to be necessary for unwinding of the double helix and progression of the replication forks (35–37). At present no function has been ascribed to CDT1 in S phase, after the pre-RCs have formed at telophase and early G1. Previous reports have shown that CDT1 is not required for keeping the MCM proteins on chromatin once the pre-RCs have been assembled (7–9, 38–40). CDT1 is expressed throughout the cell cycle, but levels fluctuate, due to proteasome degradation, being high in G1, low in S, and high again at the G2–M transition, when it is stabilized by the interaction with Geminin (38, 41).

Although CDT1 is not required for keeping the MCM proteins on chromatin once the pre-RCs are assembled (7–9, 38–40), CDT1 is degraded and DNA replication impaired when DNA is damaged during late G1 (42–45). Intriguingly, we have previously noticed that a fraction of CDT1 is stabilized during S phase and protected from proteasome degradation (41). Based on these and other observations we hypothesized that CDT1 has a role beyond pre-RC formation. In agreement with this, we demonstrate here that CDT1 binds to CDC7 and recruits CDC45 to chromatin during S phase.

EXPERIMENTAL PROCEDURES

Plasmids, siRNA, and Inhibitors—Human CDT1 was cloned as FLAG-tagged and HA-tagged versions in pcDNA3 and

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5 The abbreviations used are: pre-RC, pre-replicative complex; pre-IC, pre-initiation complex; post-RC, pre-replicative complex; siRNA, small interference RNA; HA, hemagglutinin; GST, glutathione S-transferase; CSKT, CSK-Triton; BrdUrd, bromodeoxyuridine; FACS, fluorescence-activated cell sorting.
In Vitro Kinase Assays—For in vitro kinase assays exponentially growing U2OS or HeLa cells, transfected with the indicated plasmids, were lysed with the following buffer: 50 mM Hepes/KOH, pH 7.6, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, and protease inhibitors.

DNA Synthesis—Cells synchronized in mitosis were released into G1, treated with the indicated siRNAs, and blocked at the G1/S transition by 12 h of aphidicolin treatment. After release from aphidicolin, cells were treated at the indicated time with 5 μCi/ml [methyl-3H]thymidine (GE Healthcare) for 30 min. The cells were subsequently washed with phosphate-buffered saline, treated with trichloroacetic acid 5%, and washed twice with EtOH. Dried cells were lysed with 1% SDS and 10 mM NaOH for 30 min. The measurement of [methyl-3H]thymidine incorporation was performed using a Packard γ-counter for liquid scintillation. Means ± S.D. were calculated.

BrdUrd Staining—Cells were treated with 33 μM BrdUrd for 30 min at the indicated times. Cells were then fixed with paraformaldehyde for 20 min, permeabilized with Triton 0.1%, stained with mouse anti-BrdUrd primary antibody and Cy3-conjugated anti-mouse IgG secondary antibody, and analyzed by indirect immunofluorescence. Nuclei were simultaneously counterstained with 4′,6-diamidino-2-phenylindole. The BrdUrd-positive nuclei were counted and plotted. Three independent counts (n = 150 cells each count) were used to calculate means ± S.D.

 RESULTS

DNA Damage-induced Degradation of CDT1 in G1: Role of CDT1 after Pre-RC Formation?—The past demonstration that the levels of CDT1 are strongly decreased in response to DNA damage after the pre-RCs are formed in G1 (42) led us to
Role of CDT1 in S Phase

FIGURE 1. DNA damage in late G1, leads to CDT1 degradation without decreasing chromatin-associated levels of MCM proteins. A, schematic representation of the experiment described in panel B. B, HeLa cells were released from mitosis (nocodazole block) and treated or not with the indicated doses of X-rays at the indicated times. The cells were lysed with CSKT buffer 8 h after mitotic release. Triton-soluble and Triton-insoluble chromatin-containing fractions were prepared. Immunoblotting analysis was then performed for the indicated proteins. Lysates from asynchronous, non-irradiated cells (As) and from synchronized, non-irradiated cells (NI) were analyzed in parallel. The lower bands in the immunoblot for CDT1 likely represent background bands (marked with an asterisk). MCM2 appears as a doublet. C, FACS analysis of the samples shown in panel B. D, schematic representation of the experiment described in panel E. E, HeLa cells were released from mitosis (nocodazole block) and 8 h later treated or not with either UV-rays (30 J/m²) or X-rays (10 Gy). The cells were lysed 3 h later with CSKT buffer and separated into Triton-soluble and Triton-insoluble chromatin-containing fractions. Immunoblotting analysis for the indicated proteins was performed. MCM2 appears as a doublet. F, HeLa cells were synchronized using a double thymidine block and released for 3 h or, alternatively, for 15 h in the presence of nocodazole. CDT1 levels were determined by immunoblotting. “2xThymidine” indicates a sample not released from the double thymidine block. A non-synchronized sample was analyzed in parallel. A background band is shown as loading control. G, HeLa cells were synchronized as in F and released for 3, 6, or 9 h in presence of, alternatively, cycloheximide, DMSO (as control) or MG132. Immunoblotting analysis for CDT1 is shown (two different exposure times). A background band is shown as loading control.

hypothesize that CDT1 could have a role during late G1 and in S phase. In agreement with the published results, we also observed a dramatic reduction in CDT1 levels following x-ray and UV treatments (Fig. 1). HeLa cells, pre-synchronized in mitosis, were treated with the indicated doses (10 or 30 Gy) of x-rays at 3 or 6 h after release (Fig. 1A). The cells were then lysed with CSKT buffer 8 h after mitotic release and Triton-soluble and Triton-insoluble chromatin-containing fractions were obtained. Immunoblotting analysis confirmed that CDT1 is strongly degraded upon DNA damage (Fig. 1B). Interestingly, MCM2 and MCM6 proteins were not degraded, either in the soluble or the insoluble fractions (Fig. 1B). FACS analysis showed the cells were synchronized at G1/S transition (Fig. 1C). HeLa cells were then irradiated with UV or x-rays during G1, at 8 h after nocodazole release and harvested 3 h later for immunoblotting analysis. Again, we observed a decrease in total and chromatin-associated CDT1 protein levels. Importantly, total and chromatin-associated MCM2 levels were unchanged (Fig. 1, D and E), suggesting that DNA damage does not affect the already formed pre-RCs.

Previously it has been suggested that the degradation of CDT1 upon DNA damage is related to the subsequent decrease of DNA replication (42), and we therefore hypothesized that CDT1 has a role beyond pre-RC formation. In agreement with pre-IC formation during S phase we first tested if CDT1 can interact with the CDC7-DBF4 complex (for brevity CDC7 complex), whose activity is required for pre-IC assembly and S phase progression. CDT1 has previously been shown to interact with the Cyclin A/CDK2 complex during S phase (48, 49). Because CDT1 interacts with the regulatory subunit of that complex, we tested if recombinant GST-CDT1 fusion protein could interact with DBF4. As shown in Fig. 2A, CDT1 can bind to DBF4 only when co-expressed with CDC7, suggesting that CDT1 binds to the catalytic subunit of the CDC7-DBF4 complex. We therefore tested if CDT1 can bind to CDC7 alone. To test this we also used two different catalytic inactive mutants of CDC7. One (D196N) mutated in an aspartic acid residue (of the putative ATP hydrolysis site) required for kinase activity (Fig. 2B and C), and the other (T376A) mutated in the threonine residue of the putative activation loop (50). As shown in Fig. 2D, CDT1 can bind to CDC7 in the absence of ectopically expressed DBF4 (Fig. 2D). However, interestingly, the binding of CDC7 to CDT1 was increased in cells co-expressing CDC7 and DBF4 suggesting that the regulatory subunit increases the affinity of the enzymatic subunit for CDT1. Alternatively, CDT1 might bind with higher affinity to activated CDC7. Both mutants were able to bind to CDT1 either alone or in combination with DBF4 similarly to wild type (Fig. 2D), suggesting that the increase in this, previous results have shown that a small pool of CDT1 is present during S phase and not affected by proteasome activity (41, 46, 47). To confirm this, we synchronized HeLa cells with a double thymidine block and released them for 3 h in the middle of S phase. Cells were harvested for immunoblotting and FACS analysis, which showed that all the cells were synchronized in the middle of S phase (data not shown). The immunoblotting analysis of the whole cell extracts confirmed that a small pool of CDT1 was present during S phase (Fig. 1F). In a parallel experiment, we showed that treatment of HeLa cells, released into S phase, with the protein synthesis inhibitor cycloheximide decreased the levels of CDT1 (Fig. 1G). As previously shown (38), CDT1 was also degraded at a high rate during S phase (as inferred by cells treated with the proteasome inhibitor MG132). Based on these results, we conclude that a small pool of CDT1 is maintained during S phase and hypothesize that CDT1 is involved in pre-IC formation in S phase.
affinity observed upon DBF4 overexpression is not due to CDC7 kinase activity per se. We also mapped the region of CDT1 involved in the binding to CDC7. As demonstrated in Fig. 2E, N-terminal-deleted CDT1 mutants did not bind to CDC7. Moreover, we confirmed the binding by gel filtration of CDT1 and CDC7 complex purified from baculovirus-infected cells. When expressed with the CDC7 complex, CDT1 eluted earlier and in the same fractions as CDC7 and DBF4 indicating that CDT1, CDC7, and DBF4 proteins are part of the same complex (supplemental Fig. S1). Taken together, we conclude that the CDC7-DBF4 complex can bind to the N terminus of CDT1 through its catalytic subunit. This N terminus of CDT1, which is conserved in multicellular eukaryotes (9, 51–53), has several important regulatory features, because it is required for its efficient recruitment to chromatin (33), the binding to Geminin (41, 54, 55) and its degradation (44, 45, 56) (Fig. 2F).

CDT1 Associates with the CDC7-DBF4 Complex in Vivo—To test if CDT1 and CDC7 could interact in vivo, we overexpressed CDT1 in 293 cells with either the CDC7 complex or the D196N mutant (Fig. 3B). Because CDC7 and CDK complexes bind to the same region of CDT1, we tested if a mutant of CDT1 unable to bind CDK (ΔCyCDT1) was impaired in binding to the CDC7 complex. To do this, we expressed CDT1 and ΔCyCDT1 mutant in 293 cells together with wt CDC7 or the D196N mutant complex. We observed that CDC7 was able to interact (even though slightly less efficiently) with ΔCy mutant (Fig. 3C). Moreover, we also noticed that DBF4 was less phosphorylated when CDC7 was co-expressed with ΔCy mutant (Fig. 3C).

CDT1 Promotes CDC45 Recruitment to Chromatin in Vivo (HeLa Cells)—The recruitment of the CDC7 complex to chromatin is a prerequisite for pre-IC formation and DNA replication. To test if CDT1 is required for the recruitment of CDC7 to chromatin and involved in pre-IC formation during S phase, we expressed the pre-IC component CDC45 at low levels (see “Experimental Procedures”) together with CDT1 either alone or in combination with CDC7 and DBF4 (Fig. 4, A and B). The transfected cells were then synchronized at the G1/S transition with a double thymidine block. Lysates were prepared 1 h after release from the second thymidine block and analyzed by immunoblotting for the levels of CDC45 protein associated with the chromatin. Because we were also concerned about the stability and levels of the other proteins during S phase progression (see “Results” and “Discussion” below) we treated the cells with protein synthesis inhibitors cycloheximide at the time of the first harvesting (i.e. 1 h after the release from the second thymidine block) and then prepared lysates at intervals of 3 h.

Strikingly both the expression of CDT1 and the CDC7 complex resulted in increasing levels of CDC45 on chromatin, whereas the levels in the soluble fraction were only mildly affected (Fig. 4, A and B, correspond to the soluble and chromatin-containing fractions, respectively). The co-expression of CDT1 with the CDC7 complex led to a further increase of the levels of CDC45 on chromatin, suggesting that both components are limiting the recruitment of CDC45 to chromatin. The expression of D196N mutant complex led to results similar to those obtained with the wild-type CDC7 complex suggesting agreement with the notion that the onset of re-replication is dependent on the cell type and the expression levels (34, 57). As shown in Fig. 3A, CDT1 binds both wild-type and kinase-inactive mutants of CDC7. Moreover, CDT1 can also associate with endogenous CDC7 (Fig. 3B). Because CDC7 and CDK complexes bind to the same region of CDT1, we tested if a mutant of CDT1 unable to bind CDK (ΔCyCDT1) was impaired in binding to the CDC7 complex. To do this, we expressed CDT1 and ΔCyCDT1 mutant in 293 cells together with wt CDC7 or the D196N mutant complex. We observed that CDC7 was able to interact (even though slightly less efficiently) with ΔCy mutant (Fig. 3C). Moreover, we also noticed that DBF4 was less phosphorylated when CDC7 was co-expressed with ΔCy mutant (Fig. 3C).

CDT1 Associates with the CDC7-DBF4 Complex in Vivo—To test if CDT1 and CDC7 could interact in vivo, we overexpressed CDT1 in 293 cells with either the CDC7 complex or the D196N complex and immunoprecipitated CDT1 using a CDT1-specific monoclonal antibody. In our experimental conditions CDT1 overexpression did not lead to re-replication, which is in
that CDC7 kinase activity is not required for the recruitment of CDC45 to chromatin. We also noticed that CDT1 was able to recruit the CDC7 complex to chromatin. As expected, a mutant of CDT1 deleted of the N-terminal 190 residues and unable to bind the CDC7 complex was significantly less competent in recruiting the CDC7 complex and CDC45 to chromatin. This mutant of CDT1 was also impaired in binding to chromatin and, even if expressed similarly to wt protein, much less stable upon cycloheximide treatment. We obtained very similar results after transfecting a different ratio of the DNA for the different proteins (supplemental Fig. S2).

**CDT1 Promotes CDC45 Recruitment to Chromatin in Vivo**

(U2OS Cells)—We corroborated the previous results by using another cell line. The same DNA replication factors were expressed in U2OS osteosarcoma cell lines. As before, we expressed CDC45 protein at very low levels. The transfection of exogenous CDC45 was necessary to evaluate the effect of increased levels of CDT1 and CDC7 complex, because the efficiency of transfection under these conditions was low both in HeLa and U2OS cells. The cells were synchronized as before at the G1/S transition with a double thymidine treatment, and cells were released for 1 h before harvesting and lysis (Fig. 5). Cells were then separated in two parts, one half used for the preparation of whole cell extracts and the other half used for the fractionation with CSKT buffer. The same relative amounts of each fraction were then loaded on a gel, and immunoblotting analysis was performed. Total levels of exogenous CDC45 protein are similar in the different conditions, but, as previously observed, the distribution of CDC45 protein between the soluble and the chromatin-containing fractions changed significantly when CDT1 and CDC7 complex are overexpressed, either alone or simultaneously (Fig. 5). Interestingly, we noticed that CDT1 mobility was decreased upon overexpression of the CDC7 complex, suggesting that CDT1 might be a substrate of CDC7 kinase in vivo. Moreover, as we previously observed, CDC7 associated to chromatin was increased upon overexpression of CDT1. We also observed that protein levels of CDT1 were increased upon overexpression of the CDC7 complex both in HeLa and U2OS cells (see “Discussion”). When we performed the same experiment in non-synchronized cells we obtained similar, but less striking results, indicating that the recruitment of CDC45 is facilitated during S phase (data not shown).

**CDC7 Regulates the Levels of CDT1 during S Phase**—To investigate if CDC7 regulates the binding of CDT1 to chromatin we expressed low amounts of CDT1 either alone or with the wild-type (CDC7) or kinase-inactive (D196N) complexes. Cells were synchronized at the G1/S transition, and, after release, samples were collected at different times during S phase after treatment with cycloheximide. Cell lysates were separated into Triton-soluble and Triton-insoluble fractions and used for immunoblotting analysis (Fig. 6, A and B). The expression of both CDC7 and D196N complexes led to a significant increase in soluble CDT1. Moreover, the expression of the
Inhibition of CDT1 Expression in G₁ and S Phases Reduces DNA Synthesis—Because CDT1 promotes recruitment of CDC7 complex and CDC45 in S phase, we speculated that CDT1 was required for DNA replication, also after the pre-RCs have been formed. To test this, we depleted CDT1 protein in the short period between the formation of the pre-RCs (occurring in late mitosis and early G₁) and the onset of S phase. We therefore tested siRNA for CDT1 in a short time scale and observed that CDT1 was depleted very fast after siRNA addition (supplemental Fig. S3). We therefore inhibited CDT1 expression in U2OS cells released from a mitotic block (see “Experimental Procedures” for details), and 10 h after exit from mitosis (i.e. at the G₁/S transition) we lysed the cells and separated the soluble fraction from the chromatin-containing fraction (Fig. 7A). CDT1 depletion led to decreased levels of CDC45 and RPA32 proteins on chromatin (Fig. 7B). The levels of MCM5 (data not shown), MCM6, and MCM7 proteins on chromatin were unaffected (Fig. 7B), whereas the levels of MCM2 protein were slightly decreased. We also performed FACS analysis to evaluate the equal synchronization of the two cell populations (i.e. treated with control or siRNA for CDT1) at 8 and 12 h after exit from mitosis (Fig. 7C). These results suggest that depletion of CDT1 during G₁ phase does not lead to significant changes in the pre-RC levels on chromatin, but impairs pre-IC assembly. This is in contrast to the strong decrease of MCM protein on chromatin observed upon depletion of CDT1 during mitosis (29, 41) (and supplemental Fig. S4). The likely reason for this is that pre-RCs are almost completely loaded by the time the expression of CDT1 is affected by siRNA in middle G₁ phase (supplemental Fig. S5).

To test if also DNA replication was inhibited upon similar conditions, we measured the global levels of DNA synthesis. To this purpose, we transfected HeLa and U2OS cells with siRNA for CDT1 after mitotic exit as before. At this time, pre-RCs are formed as evidenced by loading of MCM2 and MCM6 to chromatin (supplemental Fig. S5). To lengthen the period of siRNA incubation and to better synchronize the cells we used aphidicolin to arrest the cells at the G1/S transition (i.e. G₁/S transition) we lysed the cells and separated the soluble fraction from the chromatin-containing fraction (supplemental Fig. S3). We therefore inhibited CDT1 expression after pre-RC formation led to a reduction of global DNA synthesis (Fig. 7E).

Because we observed a slight decrease of MCM2 protein upon these conditions (see Fig. 7B) and though pre-RCs are formed very early during G₁, we wanted to be sure to deplete CDT1 protein only after complete pre-RCs loading on chromatin. For this reason we inhibited CDT1 expression during S phase by treating HeLa cells, immediately after release from a double thymidine block, with siRNA for CDT1 and control. We then labeled with BrdUrd for 30 min, 5 h, or 12 h after siRNA treatment (Fig. 7F). 5 h after entering S phase, the inhibition of CDT1 expression led to a small, but not significant decrease in the number of BrdUrd-positive cells, which may be due to inefficient inhibition of CDT1 expression at this time. In contrast, a significant increase in BrdUrd-positive cells was observed at 12 h, when the majority of control cells were in G₂ phase (Fig. 7, G and H). The increase of the number of BrdUrd-positive cells

CDC7 complex also resulted in a significant increase in CDT1 levels on chromatin, which however decreased during S phase. Interestingly, the levels of CDT1 on chromatin increased upon expression of D196N complex and remained constant throughout S phase. The increase of CDT1 levels required both subunits of the kinase complex, because no change in CDT1 levels was observed if D196N or DBF4 were expressed alone. Because in our transfection conditions the expression of D196N did not lead to detectable cell cycle effects (data not shown), we could rule out the possibility that CDT1 was retained on chromatin due to a cell cycle block. These results suggest that CDT1 could also be loaded on chromatin during G₁, stabilized by the CDC7 complex in early S phase, and later displaced from chromatin and pre-RC complexes by the kinase activity of the CDC7 complex. In this way, CDC7 could have a role both in the formation of the pre-IC and in its later inactivation.
Role of CDT1 in S Phase

FIGURE 6. CDC7 regulates CDT1 protein levels on chromatin during S phase. HeLa cells were transfected with low amounts of a CDT1 expression plasmid with or without expression plasmids for the indicated proteins and synchronized in S phase as in Fig. 4. After release from the second thymidine block the cells were treated with cycloheximide starting from 1 h after release (t0). Cell lysates were treated in such a way to separate the Triton-soluble fraction (A) from the Triton-insoluble chromatin-containing fraction (B) and analyzed by immunoblotting analysis for the indicated proteins. A non-transfected sample (NT) was analyzed in parallel. DBF4 co-expressed with kinase active CDC7 appears shifted on gel (i.e. lower electrophoretic mobility) due to hyperphosphorylation. In the immunoblot for DBF4 in A a background band is marked with an asterisk. L, indicates long exposure time. Tubulin and Lamin B are shown as loading controls for the soluble and chromatin-containing fractions, respectively.

FIGURE 7. Depletion of CDT1 in late G1, and S impairs DNA replication. A, outline of the experimental protocol for panel B (see text for details). B, U2OS cells were treated with the indicated siRNAs after release from nocodazole arrest. After re-addition of serum (5 h after nocodazole release) the cells were lysed at 10 h after mitotic exit and separated into a soluble and a chromatin-containing fraction. Immunoblotting analysis for the indicated proteins is shown. C, FACs analysis of the cells treated as in B and harvested at 8 and 12 h after release from mitosis are shown. D, HeLa and U2OS cells were arrested in mitosis by treatment with nocodazole, released, and 4 h later treated with siRNA for CDT1 or control. Following 3-h incubation with serum-free oligofection mixture, serum was added, and the cells were treated with aphidicolin for additional 12 h. Cells were then released from aphidicolin block and 4 h after release were analyzed by immunoblot for DBF4 in panel B or by indirect immunofluorescence in panel C. Tubulin and Lamin B are shown as loading controls. E, percentage of samples from HeLa and U2OS cells stained for BrdUrd, and counterstained with 4',6-diamidino-2-phenylindole (DAPI, at 12 h time point). F, immunoblot for DBF4 in panel B showing slightly decreased interaction with Cyclin A in C. G, depletion of CDT1 during S phase impairs DNA replication. HeLa cells plated on gelatin-treated coverslips were synchronized at G1/S transition with a double thymidine block, released, and immediately treated with siRNA for CDT1 or control. 4 h and 30 min after the second thymidine block release the cells were labeled with BrdUrd for 30 min. Cells were then fixed with paraformaldehyde and, after staining for BrdUrd, analyzed by indirect immunofluorescence. The percentages of BrdUrd-positive cells were plotted (means ± S.D. are shown). H, representative images obtained by indirect immunofluorescence analysis of cells treated as in F and G, stained for BrdUrd, and counterstained with 4',6-diamidino-2-phenylindole (DAPI, at 12 h time point).

DISCUSSION

Here we have analyzed the role of CDT1 after pre-RC formation. Our results demonstrate that CDT1 expression is required in G1 and S phases to promote DNA replication by a mechanism that involves the formation of pre-ICs. We have shown that CDT1 binds to the S phase kinase CDC7. We have not been able to show the interaction between endogenous CDT1 and endogenous CDC7, which may be due to that the interaction of the proteins only occur transiently. However, by expression studies and siRNA-mediated depletion experiments we have demonstrated that the interaction between CDT1 and CDC7 leads to recruitment of CDC7 to chromatin and loading of CDC45. Interestingly, we have also shown that the CDC7 complex stabilizes CDT1 association with chromatin during early S phase, providing a positive-acting feedback loop. Our results based on the use of kinase-inactive CDC7 complexes suggest that CDC7 kinase activity later in S phase promote the dissociation of CDT1 from chromatin.

We have shown that CDC7 binds to CDT1 regardless of its interaction with Cyclin A, but we have also shown slightly decreased interaction between ΔCy mutant and CDC7 protein (Fig. 3C). At present, we do not know if the decrease is due to lack of binding to Cyclin A or, alternatively, to intrinsic reduced affinity between CDC7 and the mutant. Furthermore, we have shown that DBF4 protein phosphorylation is decreased when co-expressed with ΔCy mutant, suggesting that CDT1 might recruit CDK2/Cyclin A to CDC7 complex.
Additionally, the levels of CDT1 are increased upon CDC7 complex overexpression both in HeLa and U2OS cells, suggesting that there could be a competition between CDC7 and CDK complexes under certain conditions. Further work is needed to shed light on the interplay between CDT1 and CDC7 and CDK complexes.

As shown in the supplemental information (supplemental Fig. S6), CDC7 can phosphorylate CDT1 in vitro. The N-terminal portion of CDT1 (containing the CDC7 interaction domain) is required for phosphorylation (supplemental Fig. S6). The shift in CDT1 mobility upon CDC7 complex overexpression in U2OS cells (Fig. 5) suggests that CDT1 might be a substrate of CDC7 kinase in vivo. However, overexpression of CDC7 in vivo only leads to a small increase in CDT1 phosphorylation by 32P labeling in vivo (data not shown). This could suggest that either only a small fraction of CDT1 is phosphorylated in vivo or, alternatively, that CDC7 indirectly regulates the turnover of the CDT1. Future studies will be aimed at understanding how CDC7 might switch from a positive to a negative regulator of CDT1 function.

Based on our results, we propose that soluble CDT1 is destabilized/inactivated by CDK activity during S phase (and marked for degradation by ubiquitin ligases), whereas chromatin-bound CDT1 is regulated by CDC7 activity (Fig. 8). We speculate that partial stabilization of chromatin-bound CDT1 permits, by supporting pre-IC formation, the timely execution of DNA replication. Indeed, when CDT1 synthesis is inhibited during G1 or S, DNA synthesis is decreased possibly due to the firing of fewer origins of DNA replication. When CDT1 is displaced from chromatin, it is most likely targeted by CDK activity and Geminin and, as a consequence, destabilized and/or inhibited (Fig. 8). Recently, it was reported that Geminin is able to bind to CDT1 on chromatin and, in this way, it might inhibit refiring of the origins of replication in the same cell cycle (33, 34). Therefore, CDC7-dependent displacement of CDT1 from chromatin during S phase progression provides an alternative way to ensure that each origin of replication is fired only once during the same cell cycle (31, 59–62). Taken together with the results presented here, we propose that stabilization of chromatin-associated CDT1 during early S and its following degradation/inhibition during S phase progression are two events both required for cell cycle execution.

CDT1 is present in unicellular eukaryotes but lacks the N-terminal domain (residues 1–190). We speculate that metazoans exploit multiple protein interactions within the N terminus of CDT1 to optimize the duplication of their large genomes and that the N terminus of CDT1 has been selected during evolution to guarantee that each portion of the genome is replicated efficiently and only once per cell cycle. Future studies are expected to shed light on interactions of CDT1 with components of the pre-IC and on the role of CDT1 in proliferation and tumor formation.

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Role of CDT1 in S Phase

FIGURE 8. Working model. We propose that CDT1 regulates pre-IC formation as a result of interaction with CDC7-DBF4 complex and CDC45 recruitment to chromatin. During S phase CDK activity destabilizes/inactivates nucleosolic CDT1, whereas CDC7 activity regulates the displacement of CDT1 from chromatin. CDT1 is marked for degradation by the SCF-SKP2 and CUL4-DBD1-CDT2 ubiquitin ligases. CDT1 protein levels increase again at the end of G2 phase due to interaction with Geminin and protection from proteasome-dependent degradation (41). Recently, it was shown that a fraction of Geminin protein binds to chromatin-bound CDT1 during S phase (33, 34). We propose that the displacement of CDT1 from chromatin and its inhibition by interaction with Geminin are two independent ways deployed by multicellular eukaryotes to ensure that each portion of DNA is duplicated only once during the cell cycle.

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