The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase

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Running title: The human Cdt1 replication licensing factor
Summary

S-phase onset is controlled, so that it occurs only once every cell cycle. DNA is licensed for replication after mitosis in G1, and passage through S-phase removes the license to replicate. In fission yeast, Cdc6/18 and Cdt1, two factors required for licensing, are central to ensuring once per cell cycle replication. We show that the human Cdt1 homologue (hCdt1), a nuclear protein, is present only during G1. After S-phase onset, hCdt1 levels decrease, and it is hardly detected in cells in early S-phase or G2. hCdt1 can associate with the DNA replication inhibitor Geminin, however these two proteins are mostly expressed at different cell cycle stages. hCdt1 mRNA, in contrast to hCdt1 protein, is expressed in S-phase arrested cells and its levels do not change dramatically during a cell cycle, suggesting that proteolytic rather than transcriptional controls ensure the timely accumulation of hCdt1. Consistent with this view, proteasome inhibitors stabilize hCdt1 in S-phase. In contrast, hCdc6/18 levels are constant through most of the cell cycle and are only low for a brief period at the end of mitosis. These results suggest that the presence of active hCdt1 may be crucial for determining when licensing is legitimate in human cells.

Key words: Cell cycle, DNA replication, Licensing, Cdt1, Cdc6/18

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**Introduction**

To maintain genome integrity, initiation of DNA replication is coordinated with passage through mitosis so that replication is limited to once every cell cycle. Cell cycle progression is globally regulated by Cyclin Dependent Kinases (CDKs) required at both S-and M-phase. The mitotic CDK activity, essential for M-phase entry, has an additional role in preventing G2 cells from re-initiating S-phase (1,2). The re-setting to G1 allowing the initiation of S-phase is achieved by inactivation of the mitotic CDK, which normally happens at the end of mitosis. When CDK activity is inappropriately inhibited in G2, however, cells are reset to initiate S-phase without entering into mitosis (3-7). Thus, CDK activity is required for ensuring that replication is limited to once per cycle.

The timely initiation of DNA replication is controlled through a process (called licensing), which ensures that only after passage through mitosis does chromatin become competent for a new round of replication (8). Studies mainly in the budding and fission yeasts and with *Xenopus* egg extracts, have lead to a model of how licensing occurs. Licensing involves the stepwise formation of pre-replicative complexes on chromatin at the end of mitosis (9-12). The Origin Recognition Complex (ORC) associates with chromatin throughout the cell cycle and, at least in the yeasts, marks origins of replication, where it acts as a landing pad for the formation of pre-replicative complexes (13, 14). When CDK activity drops at the end of M-phase, Cdc6/18 associates with origins, and loads the MCM complex onto chromatin (15-18), licensing DNA for replication. Cdt1, a newly identified licensing factor (19-22), acts at a similar step to that of Cdc6/18, and is required for the loading of MCM proteins onto chromatin (20, 21, 23). Cdt1 is conserved from fission yeast to mammalian cells, though no
good homologue has yet been reported for budding yeast (22, 24). At the G1/S transition, the licensed origins are activated by two protein kinases, the CDK-cyclin and Cdc7-Dbf4 kinase, and the loading of Cdc45 and DNA polymerase alpha onto origins, leading to the initiation of DNA replication (25-27).

In the budding and fission yeasts, the levels of Cdc6/18 are tightly controlled through the cell cycle, both at the transcriptional and post-translational level, ensuring that the protein only accumulates in G1. This control is essential for once per cell cycle replication in fission yeast, as ectopic high-level expression of Cdc18 leads to continued DNA synthesis in the absence of mitosis (28, 29). Cdc6 in budding yeast and Cdc18 in fission yeast are phosphorylated by CDKs, and are thereby targeted for ubiquitin dependent proteolysis both at S-phase and in Mitosis (30-33). Fission yeast Cdt1 (SpCdt1) is also tightly controlled through the cycle being expressed only in G1 cells (20). The Cdc10 G1/S transcriptional complex regulates SpCdt1 mRNA expression (19), similar to fission yeast Cdc18 (34). Whether SpCdt1 is regulated post-translationally by phosphorylation and/or proteolysis remains unknown. The timely expression of SpCdt1 appears important for once per cell cycle, as its ectopic expression potentiates the Cdc18 induced rereplication (20). Other putative targets mediating the CDK block to rereplication are Origin Recognition Complex subunits which are differentially phosphorylated through the cell cycle (35, 36) and the MCM proteins, which dissociate from chromatin as DNA replication proceeds (37-39). In budding yeast, one of the MCM subunits, Mcm4, is excluded from the nucleus when phosphorylated by the CDK Cdc28 (40, 41).

In contrast to the fission and budding yeasts, mammalian Cdc6/18 is less tightly controlled
through the cell cycle. While the Cdc6/18 gene is a target of E2F (42, 43), Cdc6/18 protein levels are stable for most of the cell cycle, being low only for a brief period at the end of mitosis (42, 44-48). APC-dependent proteolysis of Cdc6/18 at M phase exit may have a role in inhibiting premature licensing in mammalian cells (47). After the onset of S-Phase, soluble Cdc6/18 is phosphorylated by CDKs and is exported to the cytoplasm (49-51). Chromatin bound Cdc6/18 however appears constant for most of the cell cycle (45, 46, 48), suggesting that licensing is not primarily controlled by the presence of Cdc6/18 on chromatin. In vertebrates, in addition to CDK regulation, licensing is negatively controlled by Geminin, a protein expressed from S-phase to the end of mitosis. Ectopic expression of Geminin in a Xenopus in vitro replication system inhibits MCM loading onto chromatin (52). It was recently shown that Geminin can form a complex with Cdt1, both in Xenopus and human cell extracts (53, 54), suggesting that Geminin inhibits licensing by binding to and inactivating the MCM loading factor Cdt1. Geminin is targeted for proteolysis at the end of mitosis by the Anaphase Promoting Complex, and its degradation is essential for the subsequent licensing of chromatin for replication (52).

In this report we characterize the human homologue of Cdt1 (hCdt1) and show that, unlike hCdc6/18, hCdt1 protein levels are tightly controlled so that hCdt1 is only present in G1, when licensing takes place, while it disappears after S-phase onset. Proteolysis, rather than transcription, appears to be primarily responsible for the periodic accumulation of hCdt1. hCdt1 can form a complex with the licensing inhibitor Geminin, however these two proteins are not co-expressed in cells for most of the cell cycle.

Experimental Procedures

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**Cell culture**

HeLa, KB and Cos7 cells were grown in DMEM with 10% FBS. HeLa cells were synchronized in early S-phase by a double thymidine block (2mM) or in metaphase by nocodazole treatment (0.4µg/ml) after releasing from a double thymidine block. For transient expression in Cos7 cells, LIPOFECTAMINE (Gibco BRL) was used following the manufacturers instructions. To inhibit proteasome or protease, cells were cultured in the presence of proteasome inhibitors (MG-132, PSI (proteasome inhibitor-I), and lactacystin, all used at 20µM, Calbiochem) or calpain protease inhibitor, ZLLH (10µM, Peptide Institute Inc.). To label proteins with 35S, cells were incubated in methionine-free DMEM with 3% dialyzed calf serum for one hour and then in the same medium containing 100 µCi/ml of 35S-methionine (Tran35S-label, ICN or L-35S-methionine, Amersham/Pharmacia biotech) for one hour. For pulse-chase experiment, cells were chased with normal 10% FBS-DMEM medium supplemented with 150mg/L methionine.

**Cloning of human Cdt1**

RT-PCR was used to amplify part of the hCdt1 cDNA (PCR-hCdt1-C), using a primer set based on the randomly sequenced human cDNA AF070552 (5’ primer1 GGGGGATCCTACAAGTACCAGGTGCTGGC and 3’ primer2 GGGAAGCTTGGGGAAAGGAAGACCAAAGTG) and subcloned into the TA vector pCR2.1. Sequencing of the amplified fragment identified two nucleotide insertions when compared to the database entry, resulting in one continuous Open Reading Frame encoding the C-terminal part of hCdt1. This clone was used to screen a lambda gt10 human cDNA library and isolate a phage encoding the N-terminal part of the hCdt1 cDNA. (hCdt1-N14).

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The insert was isolated by EcoRI digestion and cloned into pBluescript. The two fragments (PCR-hCdt1-C and hCdt1-N14) were combined at the internal BglII site to construct a 1.9 kb cDNA encoding a 546 amino acid hCdt1 protein (pBlue-hCdt1). Another lambda phage clone was isolated, hCdt1-C38, which encodes for the C-terminal part of the hCdt1 Open Reading Frame and extends into the 3’ untranslated region until the polyA tail. When the three sequences (hCdt1-N14, PCR-hCdt1-C and hCdt1-C38) were combined, a cDNA of 2.74kb was generated, which has been deposited in the databases (entry AB053172) as a full length hCdt1 cDNA. This is longer by 816 bp from a hCdt1 cDNA previously reported based on EST and cDNA sequences in the databases (53). To express hCdt1 in mammalian cells, an XbaI-HindIII fragment from pBlue-hCdt1 was inserted into the mammalian expression vector pcDEB delta (55) digested with the same enzymes.

Other plasmids

pcDNAmyc-hCdc6/18 is as described (49). hGeminin was PCR amplified from a lambda cDNA library with primers: primer3 GGGGGATCCATGAATCCCAGTATGAAGC AG and primer4 GGGGAGCTTTTCATATACATGGCTT TGCATCCG, containing 5’BamHI and 3’HindIII sites respectively. The amplified product digested with BamH1 and HindIII was cloned into pcDEBdelta at BglII-HindIII. To express hGeminin with a T7 tag at the N-terminus, hGeminin cDNA was amplified with primer5 GGGGTCGACCCATGAATCCCAGTATGAAGC AG and primer4, cut with SalI-HindIII and cloned into pcDEBdelta-T7(55).

Antibodies, immunofluorescence, immunoprecipitation, Western and Northern blotting

To make anti hCdt1 antibody, a SmaI-HindIII fragment encoding hCdt1 from amino acid

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392 to the end was cloned into the pQE32 vector. The His tagged hCdt1(392-end) protein, purified on Nickel resin and then on SDS-PAGE was injected into two rabbits. The antibodies were affinity purified on a membrane with immobilised GST-hCdt1(392-end) protein, which was expressed by cloning the above SmaI-HindIII fragment into a pGEX vector.

For Western blotting, total cell lysates were prepared by lysing cell pellets directly in SDS-PAGE loading buffer and antibodies were used at the following dilutions: anti-hCdt1, 1:1500; anti-hCdc6/18 (UP biology and Santa Cruze), 1:1000; anti hGeminin (Santa Cruz), 1:1000; anti cyclin B, 1: 2000; anti-Cyclin A, 1:2000; anti-Cyclin E (Santa Cruz), 1:2000.

For immunofluorescence, HeLa or KB human cells were fixed in 3.8% formaldehyde for 10 min, or in methanol at -20°C, washed twice with phosphate buffered saline (PBS), and permeabilized with 0.3% TritonX-100 in PBS. The cells were treated with a blocking buffer (3% BSA 10% goat serum in PBS) for 30 min and incubated with primary antibody in blocking buffer. The following primary antibodies were used: anti-hCdt1 (1:200), anti-hCdc6/18 (Santa Cruz) 1:200, anti-fibrillarin (Cytoskeleton) 1:10, anti-p80 coilin (a gift from A. Lamond) 1:50, anti-PML (a gift from A. Lamond) 1:10, anti-Geminin (Santa-Cruz) 1:200. Cells were washed with PBS containing 0.1% tween-20 three times, and incubated with the following secondary antibodies: Texas-Red conjugated goat anti-rabbit antibody and FITC-conjugated goat anti mouse antibody for standard fluorescent microscopy or Alexa546 conjugated goat anti-mouse and Alexa 488 conjugated goat anti-rabbit antibodies for the colocalizations of hCdt1 with fibrillarin, coilin and PML, Alexa 488 conjugated goat anti-rabbit and Alexa 568 conjugated goat anti-mouse antibodies for hCdt1 and hCdc6/18 colocalizations and Cy3 conjugated sheep anti-rabbit and Alexa 488 conjugated donkey anti-goat antibodies for hCdt1 and hGeminin colocalizations. After washing, DNA was stained
briefly with Hoechst 33258. A Zeiss Confocal microscope was used to assess co-localizations.

For immunoprecipitation, whole cell extract was prepared by lysing cells in buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.5% NP-40, 1 mM DTT, and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 10µg/ml of pepsatatin A, leupeptin, and aprotinin).

A multi-tissue Northern (Clontech) was hybridized with a radiolabelled EcoRI-HindII fragment containing the complete coding region of the hCdt1 cDNA and a BamHI-HindIII fragment of the PCR amplified hGeminin cDNA (see above).

Results

Isolation of a human cDNA coding for a 65 kDa protein homologous to Cdt1

Database searches revealed a randomly sequenced human cDNA (Acc No AF070552, see also 22) coding for a polypeptide similar to the carboxy-terminal two thirds of *Xenopus*, *Drosophila* and fission yeast Cdt1. This sequence was PCR amplified and used as a probe against a lambda cDNA library. A clone coding for a polypeptide similar to the N-terminus of *Xenopus* Cdt1 was isolated and combined with the PCR amplified cDNA and a lambda clone extending to the 3’ untranslated region, to generate a cDNA of 2.74 kb, whose complete sequence was determined (see Experimental Procedures, deposited in the databases under entry AB053172). While our manuscript was in preparation, a cDNA sequence coding for the human Cdt1 homologue was compiled from sequences in the nucleotide databases as a combination of the randomly sequenced cDNA AF070552 with an EST database entry (53). The cDNA we cloned is extended at the 3’ end by 816 bp and has a different 5’ untranslated
region as compared to this previously reported cDNA sequence, while both contain the same open reading frame. The complete cDNA we cloned encodes a protein of 546 amino acids (60.4 kDa estimated molecular weight, hereafter referred to as hCdt1) showing 45% identity to *Xenopus* Cdt1. The C-terminal part of hCdt1 shows homology to all Cdt1 family members while the N-terminal part is more divergent (for example, the identity to *Xenopus* Cdt1 is 29% and 64% for the N- and C-terminal parts of the protein respectively, Figure 1A). The N-terminal part of hCdt1 contains a consensus site for CDK phosphorylation and a Cdk2-cyclin binding motif, as well as several TP/SP putative phosphorylation sites, suggesting that the N-terminus may become phosphorylated.

Northern blotting on total or poly A (+) RNA from exponentially growing or S-phase arrested HeLa cells using a probe from the cloned hCdt1 cDNA identified two hCdt1 mRNAs, with an approximate length of 3 and 4 kb (Figure 1B). The estimated size of the 3 kb, more abundant mRNA species is in good agreement with the size of the cDNA we identified (2.74 kb). Neither the relative expression of the two mRNAs nor the total levels appeared different between exponentially growing and S-phase arrested cells.

A multi-tissue Northern was used to assess the levels of hCdt1 expression in different human tissues (Figure 1B, right panels). The levels of human Geminin, a proposed inhibitor of S-phase entry, which is believed to act through inhibition of Cdt1 (53, 54), are shown for comparison. The levels of hCdt1 mRNA show great variation in different tissues, with highest levels in the rapidly proliferating thymus and testis and very low levels in peripheral blood leukocytes. The relative levels of the two hCdt1 mRNAs were similar in all tissues examined. We conclude that hCdt1 mRNA is expressed in cultured human cells and in tissues which

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contain a high percentage of proliferating cells.

Anti-hCdt1 antibodies were raised against the C-terminus of the protein expressed in bacteria. Affinity purified anti-hCdt1 antibodies detect a protein of 65 kDa apparent molecular weight in HeLa and KB total cell extracts (Figure 1C). A protein of the same size was over-produced when the hCdt1 cDNA we isolated was transfected into Cos7 cells. Similar results were obtained with an antibody raised in a different rabbit, while pre-immune serum from both rabbits gave no signal (data not shown). We conclude that the cDNA cloned codes for full-length hCdt1, which is specifically detected by the antibodies we raised.

**hCDT1 localizes to the nucleus of G1 cells and is concentrated in the nucleoli**

The cellular localization of hCdt1 was examined by immunofluorescence using our affinity purified anti-hCdt1 antibodies (Figure 2A). Approximately 50% of cells in a population of asynchronously growing HeLa cells showed nuclear staining. The other half of the population showed no detectable hCdt1 staining. Similar results were obtained using KB cells (data not shown). The brightness of the nucleus was variable among stained cells, while brighter staining was observed in subnuclear regions. In order to investigate what these subnuclear regions are, we carried out co-localization experiments in HeLa cells using antibodies specific for fibrillarin (a nucleolar marker), p80 coilin (a marker of Cajal/coiled bodies) or the PML (promyelocytic leukemia) protein, a marker of PML bodies. While no specific localization of hCdt1 in either Cajal bodies or PML bodies was detected (data not shown), the subnuclear regions which stain brightly for hCdt1 also stained with the anti-fibrillarin
antibody and therefore correspond to the nucleoli (Figure 2B, confocal microscopy images).

We used antibodies against two other factors implicated in licensing, hCdc6/18 and Geminin, to determine whether these factors are expressed in the same cells as hCdt1 in an asynchronous population. Co-localization experiments with hCdc6/18 and hCdt1 in HeLa cells showed that cells with no hCdt1 staining had mainly cytoplasmic hCdc6/18 staining, while most cells with nuclear hCdt1 staining also stained for hCdc6/18 (Figure 3A). It has been shown that mammalian Cdc6/18 is located in the nucleus in G1 cells while a large fraction of it is exported into the cytoplasm after S-phase onset (45, 49-51). We conclude that hCdt1 colocalises with hCdc18 in the nucleus in a subpopulation of cells, which most likely correspond to G1 cells, while appears absent in cells after S-phase onset. In Figure 3B, colocalization experiments with hCdt1 and Geminin are shown. Interphase cells which stain for hCdt1 show no staining for Geminin and vice-versa. Mitotic cells could not be accurately scored for staining with either marker, as they showed high levels of background staining (data not shown). While hCdt1 was concentrated in the nucleoli of interphase cells, Geminin appeared excluded from the nucleoli. We conclude that Geminin and hCdt1 are detected in different subpopulations of interphase cells and therefore hCdt1 protein levels must be low in S-and G2-phases of the cell cycle, when Geminin has been shown to accumulate (52).

While this work was in progress, it was reported at a meeting that Geminin binds Cdt1 from Xenopus extracts (now published, 54). In order to see whether hCdt1 and human Geminin can interact when expressed together in cells, hCdt1 or Myc-tagged hCdc6/18 were co-transfected with a plasmid expressing T7-hGeminin or non-tagged hGeminin in HeLa cells. After precipitating with a T7 specific antibody, the precipitates were examined for the
presence of hCdt1 and hCdc6/18. The T7 antibody efficiently co-precipitated hCdt1 while hCdc6/18 was not detectable in the precipitate above background, suggesting that hCdt1, but not hCdc6/18, can form a complex with Geminin (Figure 4). We therefore conclude that when Geminin and hCdt1 are co-expressed in cells, they can form a complex. Our data are in agreement with a recent report showing that Geminin present in cell extracts from asynchronously growing HeLa cells is found in a complex with Cdt1 (53). Our immunolocalization data however indicate that Geminin and hCdt1 are normally expressed in different subpopulation of interphase HeLa cells and therefore the association of the majority of endogenous Geminin with hCdt1 in extracts from asynchronous cells (53) is likely to take place after cell breakage.

hCdt1, in contrast to hCdc6/18 and Geminin, accumulates in early G1 and disappears after S-phase onset

In fission yeast, Cdt1 accumulation closely mirrors that of Cdc18 as cells progress synchronously through the cell cycle, suggesting that the levels of the two proteins are controlled in similar ways (20). Our immunolocalization experiments however suggested that in human cells hCdt1 protein levels, in contrast to hCdc6/18, vary significantly through the cell cycle. In order to establish whether hCdt1 and hCdc6/18 are indeed regulated in a different way in human cells, we undertook a detailed analysis of their protein levels in synchronously dividing HeLa cells.

HeLa cells arrested in S-phase by a double thymidine block were released into a synchronous cell cycle and sampled every two hours. Cells completed S-phase by 8 hours and mitosis by Nishitani et.al.
12 hours and proceeded to a second S phase by 25 hours (Figure 5A, flow cytometry). Protein levels of hCdt1, hCdc6/18 and Geminin were determined by Western blotting. To allow an accurate assessment of cell cycle stage, the levels of cyclin A, cyclin B and cyclin E were determined in the same samples, while hOrc2, a subunit of the human origin recognition complex which is expressed at constant levels through the cell cycle, served as loading control (Figure 5A). hCdt1 is present at very low levels in cells in S-phase and G2, and starts accumulating as cells exit mitosis (as cyclin B and Geminin levels decrease). Its levels remain high through G1 and decrease again as cells enter a second S phase. In sharp contrast, hCdc6/18 protein levels are high throughout S-phase and G2, dropping in mitosis and rising again in late G1, consistent with previous studies. hCdc6/18 is expressed several hours later than hCdt1 in G1, its accumulation most closely resembling that of cyclin E. Its levels however remain high as cells undergo S-phase again.

A recent report suggested that hCdt1 and hGeminin may be coexpressed in S-phase (53). Our data however indicate that hCdt1 is at very low levels in cells arrested in S-phase by a double thymidine block. In order to address this issue more carefully, we used two more DNA synthesis inhibitors, aphidicolin (AP) and hydroxyurea (HU), which arrest cells at an earlier stage in S-phase than a double thymidine block (Figure 5B, flow cytometry). hCdt1 protein levels are low in all these S-phase blocks, indicating that hCdt1 becomes unstable soon after DNA replication starts. hCdc18 and hGeminin in contrast accumulate in all the S-phase blocks. We then followed cells that were released from the double thymidine block but were inhibited from completing mitosis by the microtubule depolymerizing drug nocodazole. As cells accumulated in the mitotic block, hCdt1 protein levels increased but hCdt1 migrated slower than in asynchronous cells, indicating that hCdt1 accumulates in a modified form in
nocodazole arrested cells. hCdc6/18 and Geminin also showed a similar accumulation in a slower migrating form in the mitotic block. As expected, cyclin B levels were high in the metaphase block.

hCdt1, hCdc6/18 and Geminin protein levels were determined in cells released from a nocodazole arrest (Figure 5C). Cyclins were used as markers of cell cycle stage, while hOrc2 served as loading control. Upon release from the block, as mitosis was completed and cyclin B and Geminin levels fell, the slower migrating hCdt1 isoform was replaced by the faster migrating form, which persisted in G1. Consistent with our previous data, hCdt1p disappeared in S-phase (as cyclin A and Geminin accumulated) and was expressed again 25 hours after release, when cells were in G1. hCdc6/18 protein levels decreased as cells completed mitosis, while hCdc6/18p peaked again (in the faster migrating form) in mid-G1 and remained high through S-phase and G2.

Our data, taken together, show that the cell cycle accumulation of hCdt1 differs significantly from that of hCdc6/18. hCdt1 is absent from S-phase cells and accumulates rapidly upon mitotic exit while hCdc6/18 is stable in S-phase, its levels are low in mitosis and accumulates significantly later than hCdt1 in mid-G1. hCdt1 is only present in cells in the period from mitotic exit to onset of S-phase, when the licensing of DNA for a further round of replication occurs. This suggests that the timely accumulation of hCdt1 may be central for ensuring once per cell cycle replication.

**hCdt1 levels are controlled by proteolysis in S-phase**

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The cell cycle changes in hCdt1 protein levels could result from cell cycle-dependent transcription or/and proteolysis. We performed a Northern blot with total RNA prepared from synchronized cells after release from a double thymidine block (Figure 6A). hCdt1 mRNA was present in cells arrested in S-phase and its amount did not change dramatically as the cell cycle proceeded. hCdt1 mRNA was also detected in cells arrested in M-phase with nocodazole, as expected from the finding that hCdt1 is produced in these cells. hCdc6/18 mRNA levels showed a similar profile through the cell cycle to hCdt1 mRNA, in contrast to their differential accumulation at the protein level. Similarly, Geminin mRNA levels did not vary significantly through the cell cycle. We conclude that hCdt1 protein levels through the cell cycle are mainly regulated post-transcriptionally.

hCdt1 protein fails to accumulate in S-phase despite the presence of its mRNA, suggesting that proteolysis may be responsible for keeping protein levels low. We therefore tested whether treating S-phase cells with proteasome inhibitors would stabilize hCdt1 (Figure 6B). A significant increase in hCdt1 protein levels was observed when cells arrested in early S-phase by a double thymidine block were treated with the proteasome inhibitors, proteasome inhibitor I, MG-132 (Figure 6B) or lactacystin (data not shown), while treatment with ZLLH (a calpain inhibitor) had no effect. In contrast, hCdc6/18 or hMcm3 protein levels were not affected by the presence of proteasome inhibitors in S-phase arrested cells. hCdt1 accumulating in S-phase cells in the presence of proteasome inhibitors appears to migrate somewhat slower than in exponentially growing cells, but is not as retarded as in nocodazole arrested cells, suggesting that hCdt1 is subject to modification in S-phase cells which differs from its modification in mitosis. Upon longer exposure, a ladder of hCdt1 bands with much retarded mobility can be detected in proteasome inhibitor treated S-phase cells, which could
correspond to ubiquitinylated intermediates. We performed a pulse-chase experiment to compare the half-life of hCdt1 in asynchronously growing and S-phase arrested cells. As shown in Figure 6C, hCdt1 is degraded more rapidly in S-phase arrested cells. We conclude that hCdt1 is targeted for proteasome dependent degradation in S-phase. This could be mediated through SCF, which is active in S-phase cells.

Discussion

We have characterized a human member of the Cdt1 family of licensing factors. Fission yeast and *Xenopus* Cdt1 family members are required for the loading of the MCM proteins onto chromatin upon mitotic exit, thereby licensing DNA for a new round of DNA replication (20, 21). hCdt1 contains all the Cdt1 family conserved domains (data not shown, 24) and is highly homologous to *Xenopus* Cdt1 (45% overall identity). The N-terminus of the protein is more divergent (29% identity to *Xenopus* Cdt1) and is also shorter than *Xenopus* Cdt1 by 80 amino acids, which accounts for the difference in size between the human and *Xenopus* proteins (65 kDa versus 75 kDa respectively). The N-terminus has several SP or TP putative phosphorylation sites as well as a consensus CDK phosphorylation site (SPXR). *Xenopus* Cdt1 is phosphorylated in metaphase of meiosis II and we observed a similar band shift in HeLa cells arrested in mitosis with nocodazole, making it likely that hCdt1 is phosphorylated in a mitotic block. hCdt1 may also be phosphorylated during S-Phase (see below).

hCdt1, like fission yeast Cdt1, localizes to the nucleus. A putative bipartite NLS can be found at the N terminus of hCdt1 (amino acid number 48 RKRAR…..RRRLR71), which may be responsible for directing hCdt1 to the nucleus. hCdt1 is more concentrated in the nucleoli. Whether the accumulation of hCdt1 in the nucleolus has any role in regulating its activity, as
shown for other cell cycle proteins (reviewed in 56), remains to be investigated. It is interesting to note in that respect that Geminin, which is believed to negatively regulate Cdt1, appears excluded from the nucleoli of interphase HeLa cells.

Licensing must be restricted to G1 so that DNA replication only takes place once in each cell cycle. In mammalian cells the levels of most initiation factors (MCM family members, Origin Recognition Complex components and Cdc6/18) appear relatively stable for most of the cell cycle. Soluble hCdc6/18 is exported to the cytoplasm in S-phase, in a process regulated by Cdk2/cyclin A, chromatin bound hCdc6/18 however persists in S and G2 (42, 44-51). How is then licensing restricted to G1? We show here that hCdt1, a protein essential for licensing in fission yeast and *Xenopus*, is only present when licensing is appropriate. The hCdt1 protein accumulates soon after cells exit mitosis and remains at high levels through G1. As cells enter S-phase, hCdt1 protein levels fall and remain low for G2 and M. hCdt1 was not detected in cells arrested in early S-phase by a number of different blocks, indicating that hCdt1 becomes unstable upon initiation of S-phase. The oscillation of hCdt1 protein levels, similar to that of fission yeast Cdc18 and Cdt1, may therefore be important for restraining origin re-firing in S-phase and G2 in human cells.

How is the timely accumulation of hCdt1 achieved? We show that hCdt1 mRNA is expressed in cells arrested in S-phase and mRNA levels do not change dramatically through the cell cycle. In contrast, proteasome inhibitors stabilize hCdt1 in S-phase, suggesting that the periodic accumulation of hCdt1 is mainly due to proteolytic control. The SCF dependent proteolytic pathway is activated at G1/S and SCF targets are marked for degradation by CDK dependent phosphorylation (57, 58). hCdt1 has a consensus site for CDK phosphorylation and
close to it, the sequence PARRRLRL which is similar to the Cyclin-Cdk2 binding site found in E2F3 (PAKRRLEL, 59, 60). The decrease in hCdt1 protein levels closely mirrors the accumulation of cyclin A, suggesting that hCdt1 may be phosphorylated by a cyclin A-CDK complex and targeted for SCF-dependent proteolysis in S-phase. Consistently, hCdt1 accumulating upon treatment of S-phase arrested cells with proteasome inhibitors has a somewhat reduced mobility in comparison to exponential cells, which could be due to phosphorylation, while a ladder of retarded bands can be detected upon longer exposure, which could correspond to ubiquitinylated intermediates.

hCdt1 levels are also inversely correlated with the levels of the licensing inhibitor Geminin. Geminin can interact with hCdt1 in extracts from human cells (53 and this study), the two proteins however do not appear to be expressed in the same cells in interphase. While binding to Geminin might target hCdt1 for degradation in human cells, it is more likely that Geminin acts in a redundant pathway to ensure that any hCdt1 escaping degradation will be inactivated by Geminin binding. Geminin accumulates in S-phase, and it was proposed that Geminin inhibition may be essential for restricting origin refiring when DNA replication is inhibited (52, 53). However our data show that hCdt1 levels decrease when cells enter S-phase, and hCdt1 is unstable in three different S-phase blocks (hydroxyurea, aphidicolin and double thymidine block) indicating that re-replication is inhibited, at least in part, by S-phase specific hCdt1 proteolysis. We show that hCdt1 and Geminin both accumulate in a mitotic block, where the presence of Geminin may be particularly important for inhibiting premature licensing. This is similar to Xenopus, where both proteins are present in metaphase arrested oocytes (21, 52, 54). hCdt1, Geminin and hCdc6/18 appear hypermodified in HeLa cells arrested in mitosis, which may also inhibit licensing until the end of mitosis. The modification
of hCdt1 in cells arrested in mitosis appears different from the modification in S-phase arrested cells treated with proteasome inhibitors and may serve a different purpose (e.g. inhibiting hCdt1 function in mitosis versus targeting the protein for degradation in S-phase).

Analysis of hCdt1 and Geminin mRNA levels in different human tissues showed that hCdt1 is highly expressed in rapidly proliferating tissues such as the thymus and testis, as expected for a factor implicated in cell cycle progression. Geminin is most highly expressed in the testis, suggesting that hCdt1 inhibition by Geminin may be particularly important in this tissue.

Cdt1 is believed to act together with Cdc6/18 to accomplish the loading of MCM proteins onto chromatin (20, 21). Our data however show that hCdt1 and hCdc6/18 are regulated in different ways. hCdc6/18 protein levels are mostly unchanged through the cell cycle and are only low for a brief period in mitosis. We show here that hCdc6/18 is expressed considerably later than hCdt1 in G1, suggesting that the G1 accumulation of hCdt1 and hCdc6/18 is not controlled by the same factors. hCdc6/18 accumulation most closely mirrors the accumulation of cyclin E and hCdc6/18 levels remain high in S-phase and G2, while hCdt1 is absent from S-phase and G2 cells and is additionally controlled by binding to Geminin.

Why would two proteins with a very similar cellular function be controlled in such different ways? One possibility is that the different modes of regulation ensure that at least one of these essential licensing factors would be correctly expressed and therefore restrain untimely licensing. Ectopic expression of Cdt1 and Cdc18 in fission yeast cells leads to rereplication. Human cells may ensure that such an untimely expression of both proteins never takes place by imposing different modes of regulation upon them. Alternatively, hCdt1 and hCdc6/18, in

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addition to their common involvement in licensing, may have other roles in the cell which remain to be identified.

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**Figure legends**

Figure 1. Human Cdt1 homologue (A) Alignment of predicted human Cdt1 with XCdt1. The bars indicate consensus phosphorylation sites by CDK. The arrow shows a sequence similar to the CDK-Cyclin binding motif. (B) Northern analysis of hCdt1. (left) PolyA(+) RNA (lanes 2 and 3: 0.7μg) or total RNA (lanes 4 and 5: 20μg) from exponentially growing cells (EX: lanes 2 and 4) or HU arrested S phase cells (S: lanes 3 and 5) were probed with hCdt1 cDNA. lane 1 shows the position of 28S and 18S rRNAs. (right) Multi-tissue Northern analysis of hCdt1 and Geminin mRNAs. Each lane contains 2 μg PolyA (+) RNA from the following tissues lane 6: spleen, lane 7: thymus, lane 8: prostate, lane 9: testis, lane 10: ovary, lane 11: small intestine, lane 12: colon, lane 13: peripheral blood leukocyte. (C) Immunoblotting. Whole cell extracts were prepared from Cos 7 cells transfected with empty vector (lane 1) or with pcDEBdelta-hCdt1 (lane 2), and human cell lines HeLa (lane 3) and KB (lane 4). 25μg of each extract was separated on SDS-PAGE and blotted with anti hCdt1 antibody.

Figure 2. hCdt1 is located in the nucleus of human cells and is concentrated in the nucleoli.

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(A) HeLa cells were fixed in paraformaldehyde, and stained with anti hCdt1 antibody (top) and Hoechst33258 (middle). The bottom panel shows a Nomarski image of the cells. (B) Methanol fixed HeLa cells were stained with an anti-hCdt1 antibody (top) and an anti-fibrillarin antibody (middle). Colocalisation of the two proteins is shown on the bottom panel (confocal microscopy images).

Figure 3. Localization of hCdt1, hCdc6/18 and hGeminin in HeLa cells

(A) Paraformaldehyde fixed HeLa cells were stained with an anti-hCdt1 antibody (top), and an anti-hCdc6/18 antibody (middle). Colocalisation of the two proteins is shown in the bottom panel. (B) Paraformaldehyde fixed HeLa cells were stained with an anti-hCdt1 antibody (top) and an anti-hGeminin antibody (middle). Colocalisation of the two proteins is shown in the bottom.

Figure 4. hGeminin associates with hCdt1, but not with hCdc6/18, when these factors are co-expressed in Cos7 cells. myc-hCdc6/18 and hCdt1 cDNAs were co-transfected into Cos7 cells with T7-hGeminin or non-tagged hGeminin. After precipitation with an anti T7 antibody, precipitates were blotted with anti T7 and anti Cdc6/18 or anti hCdt1 antibodies as indicated. 5μg of cell extract and immunoprecipitate from 125μg extract were loaded.

Figure 5. Cell cycle expression of hCdt1. (A) HeLa cells arrested in S phase by a double thymidine method were released into a synchronous cell cycle and sampled every 2 or 3 hours. (B) HeLa cells were arrested in early S by thymidine-aphidicolin (AP), thymidine-hydroxyurea (HU), or double thymidine (DT) methods, or arrested at metaphase by adding Nishitani et.al.
nocodazole 6 hrs after release from a double thymidine block and collected at the indicated times (hours after release from S). (C) HeLa cells arrested at metaphase with nocodazole were released into a synchronous cell cycle and collected at the indicated time points (hours). DNA content was analyzed by FACScan while protein levels were determined by Western blotting with the indicated antibodies. The arrows in (B) and (C) show the two bands migrating faster in interphase and slower at mitosis for each protein. The * in (B) for the Cdt1 Western marks a nonspecific band.

Figure 6. (A) Northern analysis of hCdt1 mRNA levels. HeLa cells arrested in early S by a double thymidine method were released into a synchronous culture, same as in Figure 5A, and collected at the indicated time points. Nocodazole was added to two dishes at 6 hours and cells collected at 12 and 14 hour after release from S phase. Total RNA was prepared and blotted with hCdt1, hCdc6/18 and Geminin. Staining of the ribosomal RNAs serves as a loading control. (B) Proteasome inhibitors stabilize hCdt1p in S-phase. HeLa cells arrested in S-phase were treated with the indicated inhibitors for 3, 6, 9 and 12 hours, collected and analyzed for the levels of hCdt1, hCdc6/18 and hMCM3 proteins by Western blotting. A longer exposure for hCdt1 is shown in the second panel, which allows visualization of a ladder of retarded hCdt1 bands, possibly corresponding to ubiquitinylated intermediates. Extracts from nocodazole arrested (no) and asynchronous cells (exp) were run in parallel, to allow direct comparison of the migration of hCdt1. hCdt1 in S-phase arrested cells treated for 3 and 6 hours with proteasome inhibitors has an electrophoretic mobility intermediate to the one observed in nocodazole arrested and asynchronously growing cells. Both the slower migration of the main hCdt1 band and the ladder of retarded bands disappear after longer times in the presence of inhibitors (9 and 12 h timepoints) when cells appear to be entering

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programmed cell death (ref 61 and data not shown). (C) hCdt1 has a reduced half life in S-phase arrested cells. (left panel) Anti-hCdt1 antibodies precipitate a 65kDa protein. HeLa cells were labeled with $^{35}$S-methionine as described in Experimental Procedures. Immunoprecipitates with anti-hCdt1 antibodies (lane2) and with control rabbit IgG (lane1) were analyzed on a 5-20% SDS gradient gel. The band corresponding to hCdt1 is marked with an asterisk. (right panel) Pulse-chase experiments of hCdt1. HeLa cells growing asynchronously or arrested in S-phase by a double thymidine block were grown in the presence of $^{35}$S-methionine for one hour, were then transferred to medium containing unlabelled methionine and sampled 0, 30 min and 1 hour after the transfer. Following immunoprecipitation with anti-hCdt1 antibodies, the immunoprecipitated proteins were analyzed on a 10% SDS-PAGE gel and the intensity of the band corresponding to hCdt1 (marked with an asterisk) was quantified (values shown under the corresponding lanes). The intensity of the hCdt1 band over time is plotted on the bottom part of the right panel.
Figure 1
Figure 4
Figure 6
The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase
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