Essential role of human CDT1 in DNA replication and chromatin licensing

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Summary

Formation of pre-replicative complexes at origins is an early cell cycle event essential for DNA duplication. A large body of evidence supports the notion that Cdc6 protein, through its interaction with the origin recognition complex, is required for pre-replicative complex assembly by loading minichromosome maintenance proteins onto DNA. In fission yeast and *Xenopus*, this reaction known as the licensing of chromatin for DNA replication also requires the newly identified Cdt1 protein. We studied the role of hCdt1 protein in the duplication of the human genome by antibody microinjection experiments and analyzed its expression during the cell cycle in human non-transformed cells. We show that hCdt1 is essential for DNA replication in intact human cells, that it executes its function in a window of the cell cycle overlapping with pre-replicative complex formation and that it is necessary for the loading of minichromosome maintenance proteins onto chromatin. Intriguingly, we observed that hCdt1 protein, in contrast to other licensing factors, is already present in serum-deprived G0 arrested cells and its levels increase only marginally upon re-entry in the cell cycle.

Key words: Cdt1, MCM, Pre-replicative complexes, DNA licensing

Introduction

In eukaryotic organisms DNA replication initiates from multiple origins (Cairns, 1966; Huberman and Riggs, 1968). At least three different steps are essential for origin function: first, origin DNA is recognized by the origin recognition complex (ORC); second, pre-replicative complexes (pre-RCs) containing Cdc6 and MCM proteins are built at origins; and third, origins are activated by cyclin dependent and Cdc7 kinases thus initiating bi-directional replication (Dutta and Bell, 1997). These events are tightly co-regulated with cell cycle events and multiple control pathways exist that ensure the precise and timely duplication of the genome (Diffley, 1996; Diffley, 2001). Cdt1 is a novel replication factor conserved throughout evolution. Cdt1 gene was first identified in *S. pombe* as a target of Cdc10 transcriptional regulation (Hofmann and Beach, 1994). Recent work has demonstrated that Cdt1 cooperates with Cdc18/Cdc6 in driving re-replication in G2 when the Cdc18/Cdc6 protein is overexpressed (Yanow et al., 2001) and that Cdt1 depletion prevents the binding of MCM proteins to chromatin (Nishitani et al., 2000). A requirement for Cdt1 in DNA replication has also been demonstrated in *Xenopus* and *Drosophila* (Maiorano et al., 2000; Whittaker et al., 2000). Regulation of Cdt1 function appears to be of critical importance: studies using a cell-free DNA replication system from the *Xenopus* egg have revealed that geminin, a DNA replication inhibitor and anaphase promoting complex (APC) substrate, tightly binds Cdt1 and blocks its function (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000; Tada et al., 2001). Geminin has not been found in yeast to date suggesting that Cdt1 regulation might be achieved through different mechanisms.

The human Cdt1 gene was recently isolated; hCdt1 protein fluctuates during the cell cycle in highly proliferative HeLa cells and interacts with geminin (Wohlschlegel et al., 2000; Nishitani et al., 2001). Moreover, addition of recombinant hCdt1 protein to a geminin-poisoned *Xenopus* egg extract was shown to restore the extract's ability to replicate exogenous DNA (Wohlschlegel et al., 2000). Whether this occurs simply by titrating out geminin, rather than providing activity itself, is not yet known. In this study, we investigated the role of hCdt1 in DNA replication in intact cells. We report that hCdt1 protein is present in G0 and early G1 phases of cell cycle in normal cells and that ablation of hCdt1 function causes inhibition of cellular DNA replication by blocking the licensing reaction.

Materials and Methods

Antibodies and plasmids

Anti-hCdt1 antibodies were obtained by rabbit immunization with a synthetic peptide of sequence CADLAHITARLAHQTRAEEGL. Antibodies were affinity-purified against the peptide as described (Harlow and Lane, 1988). Anti-geminin antiserum was raised against a synthetic peptide of sequence NPSMKQKQEEIKENIKNSS. Anti-Cdc6 (DCS-180) was purchased from Neomarkers, anti-cyclin A (H-432) from Santa Cruz Biotechnology, anti-BrdU from Amersham and anti-MCM2 (68676E) from Pharmingen. Human Cdt1 cDNA was obtained from Image Consortium.

Cell culture and synchronization

U2OS osteosarcoma cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). U2OS were synchronized in early mitosis by nocodazole (50 ng/ml) for 16 hours, in G1/S boundary by hydroxyurea (5 mM) or aphidicolin (5 μg/ml) for 24 hours. Normal dermal human fibroblasts (NHDF) were maintained in fibroblast basal medium (FBM,

Promocell) with 10% FCS and 1 ng/ml of human fibroblast growth factor (FGF). NHDF cells were serum starved for 72 hours in FBM plus FGF and 0.1% FCS.

Antibody microinjection and immunofluorescence

Synchronized U2OS cells on glass coverslips were microinjected with GFP encoding DNA (20 ng/μl) and either affinity-purified anti-Cdt1 antibodies (1 mg/ml) or the same antibodies preincubated for 1 hour with antigen peptide (0.5 mg/ml). An automated capillary system (Zeiss AIS 2) at a pressure of 50-100 hPa was used for nuclear microinjection. Cells were fixed in 4% paraformaldehyde.

For MCM2 binding experiments, a Histone 2B-GFP fusion expressing plasmid instead of GFP vector was used as a marker because it is resistant to Triton washes. Extraction of unbound MCM2 from nuclei was achieved with a 20 minute wash in cold CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) supplemented with 0.5% Triton X-100 and protease inhibitors before methanol fixation.

Results

Generation and characterization of anti-Cdt1 antibodies

To study the function of hCdt1, we raised antibodies against a synthetic peptide corresponding to C-terminal residues. In western blot experiments, this serum recognizes a 65 kDa band that is greatly increased in protein extracts prepared from U2OS cells overexpressing hCdt1 (Fig. 1A). A band of same molecular weight is detected in protein extracts obtained from normal human dermal fibroblasts (NHDF) after a long exposure (Fig. 1B). Both immunological reactions could be abolished when the antibodies were preincubated with antigen peptide. Thus, peptide competition experiments allowed us to unambiguously detect hCdt1 protein. Following affinity-purification the antibodies essentially recognize a single band corresponding to hCdt1 in different cell lines. Again this reaction could be blocked by an excess of competing peptide (Fig. 1C). Affinity-purified antibodies were subsequently used in all microinjection experiments.

Cdt1 protein is expressed in quiescent cells and accumulates before S phase entry

It was previously shown that hCdt1 protein fluctuates during the cell cycle in HeLa cells (Wohlschlegel et al., 2000; Nishitani et al., 2001). We further extended this observation by examining hCdt1 expression in normal non-transformed primary cells. NHDF were serum starved and stimulated by addition of fresh medium containing 10% serum, samples were taken at 4 hour intervals. FACS analysis together with cyclin A accumulation indicates that NHDF entered S-phase around 16 hours after serum stimulation and most cells had completed DNA replication after 28 hours. G0 NHDF cells contain low but detectable levels of hCdt1. hCdt1 protein increases two- to threefold in late G1, and then decreases in S-phase (Fig. 2). Geminin, a Cdt1 inhibitor, began to accumulate in S phase following the peak of hCdt1 and concomitantly with cyclin A, suggesting that hCdt1 function may be limited to a window of the cell cycle that precedes DNA synthesis. This result is consistent with observations that geminin in HeLa cells accumulates in S and G2 phases (Wohlschlegel et al., 2000; Nishitani et al., 2001). In fission yeast Cdt1 is cell cycle co-regulated with Cdc18/Cdc6 (Nishitani et al., 2000). Moreover, in *Drosophila* DUP mRNA, the Cdt1 homologue is controlled by E2F transcription factor (Whittaker et al., 2000), as is Cdc6 mRNA in human cells (Hateboer et al., 1998; Leone et al., 1998; Yan et al., 1998), suggesting that hCdt1 and Cdc6 may also be co-regulated in human cells. In these experimental conditions we observed that Cdc6 accumulates with slightly delayed kinetics compared with hCdt1. Further experiments will be necessary to determine whether hCdt1 cell cycle regulation occurs at the transcriptional or post-transcriptional level or both.

Ablation of Cdt1 function prevents DNA replication initiation

Expression timing suggests that hCdt1 might play a role in early stages of DNA replication similar to what has been observed in model organisms (Nishitani et al., 2000; Maiorano et al., 2000). We used the nuclear antibody microinjection approach to test this hypothesis. Nocodazole-treated U2OS cells were injected with highly affinity-purified anti-Cdt1 antibodies together with green fluorescent protein (GFP) encoding DNA. After microinjection, nocodazole was removed and cells were incubated in fresh medium in the presence of 5′-bromodeoxyuridine (BrdU) for a further 20 hours. Microinjected cells, which expressed GFP, were

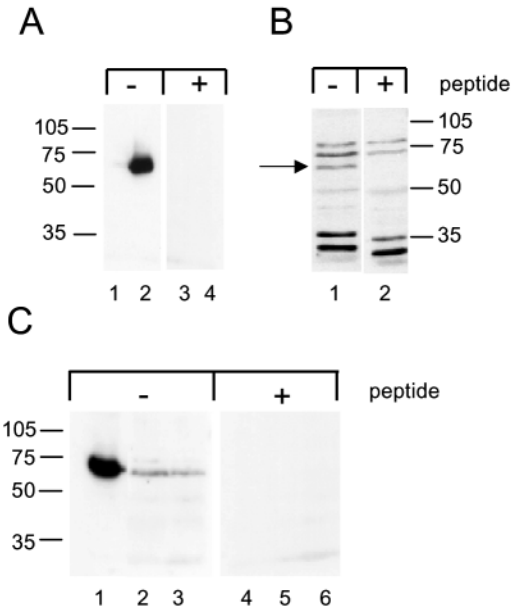

Fig. 1. Characterization of anti-Cdt1 antibodies. (A) 10 μg of protein extracts prepared from U2OS cells transiently transfected with either empty vector (lanes 1,3) or with plasmid expressing hCdt1 (lanes 2,4) were probed with anti-Cdt1 serum in the absence (lanes 1,2) or presence of competitor peptide (lanes 3,4). (B) 50 μg of protein extracts obtained from NHDF were blotted with anti-Cdt1 serum in the absence (lane 1) or presence (lane 2) of competitor peptide. The arrow indicates hCdt1 protein. (C) 50 μg of protein extracts from U2OS cells transfected with Cdt1-expressing plasmid (lanes 1,4), or from U2OS (lanes 2,5) or HeLa (lanes 3,6) cells probed with immunopurified anti-Cdt1 antibodies in the absence (lanes 1-3) or in presence of competitor peptide (lanes 4-6).

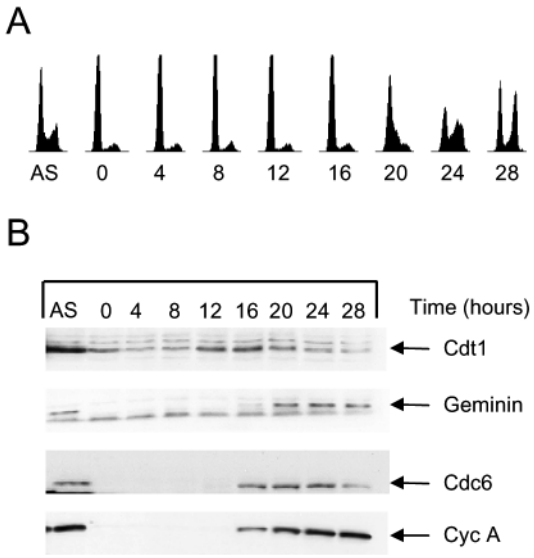

Fig. 2. Analysis of licensing factors upon re-entry into cell cycle. NHDF cells were arrested in G0 and stimulated with 10% serum to enter cell cycle. Samples were taken at the indicated times after serum stimulation. (A) DNA content was measured by FACS. (B) Total cell extracts were prepared and hCdt1, geminin, Cdc6 and cyclin A protein levels were analyzed by western blot.

assayed for their capability to incorporate BrdU into DNA by indirect immunostaining. As a control the same anti-Cdt1 antibodies preincubated with antigen peptide were injected. hCdt1 ablation clearly caused inhibition of S-phase. Indeed, almost 80% of cells microinjected with anti-Cdt1 antibodies failed to initiate DNA synthesis. By contrast, only 28% of control cells microinjected with anti-Cdt1 antibodies pre-bound to peptide did not replicate their DNA (Fig. 3). Inhibition of DNA synthesis in cells microinjected with anti-Cdt1 antibodies could be caused by the incapability of these cells to properly execute mitosis. However, we observed that at the time of cell fixation, 20 hours post release, the chromatin of microinjected cells was decondensed; thus excluding the possibility of a late mitotic block. Moreover, in a parallel experiment we found that at 12 hours post-release, cells microinjected with anti-Cdt1 antibodies and cells microinjected with anti-Cdt1 antibodies neutralized with antigen peptide had degraded cyclin B1 with approximately the same efficiency as surrounding cells that were not injected (data not shown).

Ablation of protein function through antibody microinjection can be used to define the execution point of a given protein during cell cycle (Pagano et al., 1992; Ohtsubo et al., 1995). DNA replication is a two-step process: first, pre-replicative complexes are assembled at origins and subsequently converted into active replication forks (Diffley, 1996). To determine whether hCdt1 function is required for either one of these two mechanisms, cells that had been blocked in early S-phase with hydroxyurea (HU) were microinjected with anti-Cdt1 antibodies. HU-treated cells arrest with a nearly G1 DNA content when measured by FACS (data not shown); however, origin licensing has already

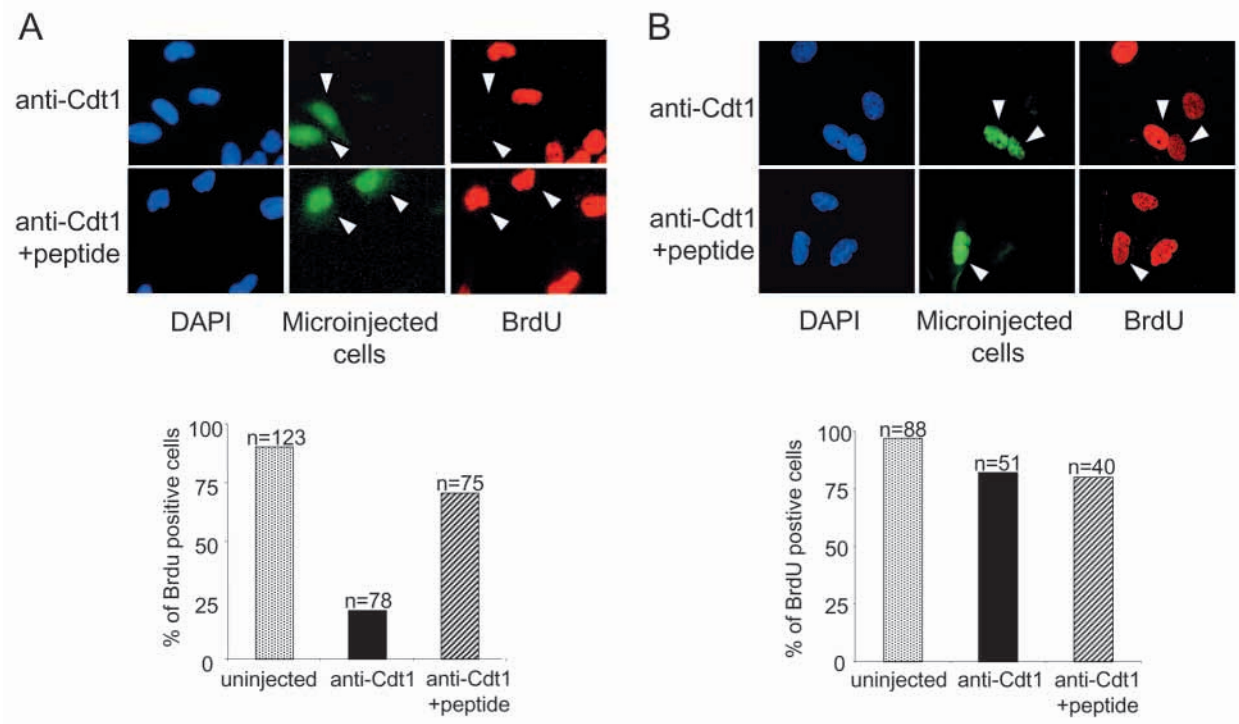

Fig. 3. hCdt1 function is essential for DNA replication initiation. (A) Nocodazole treated, G2/M arrested U2OS cells were microinjected with anti-Cdt1 (black bars) or anti-Cdt1 antibodies neutralized with antigen peptide (dashed bars). Microinjected cells (white arrows) also express GFP. 20 hours after nocodazole release in the presence of BrdU, cells were fixed and scored for BrdU incorporation. The graphs summarize the number of BrdU-positive cells in each sample. The number of scored cells is indicated. Original magnification ×63. (B) HU-treated, S-phase arrested cells were injected as in A. Cells were released from HU into fresh medium for 20 hours. BrdU was present for the first 7 hours after HU release. Original magnification ×630.

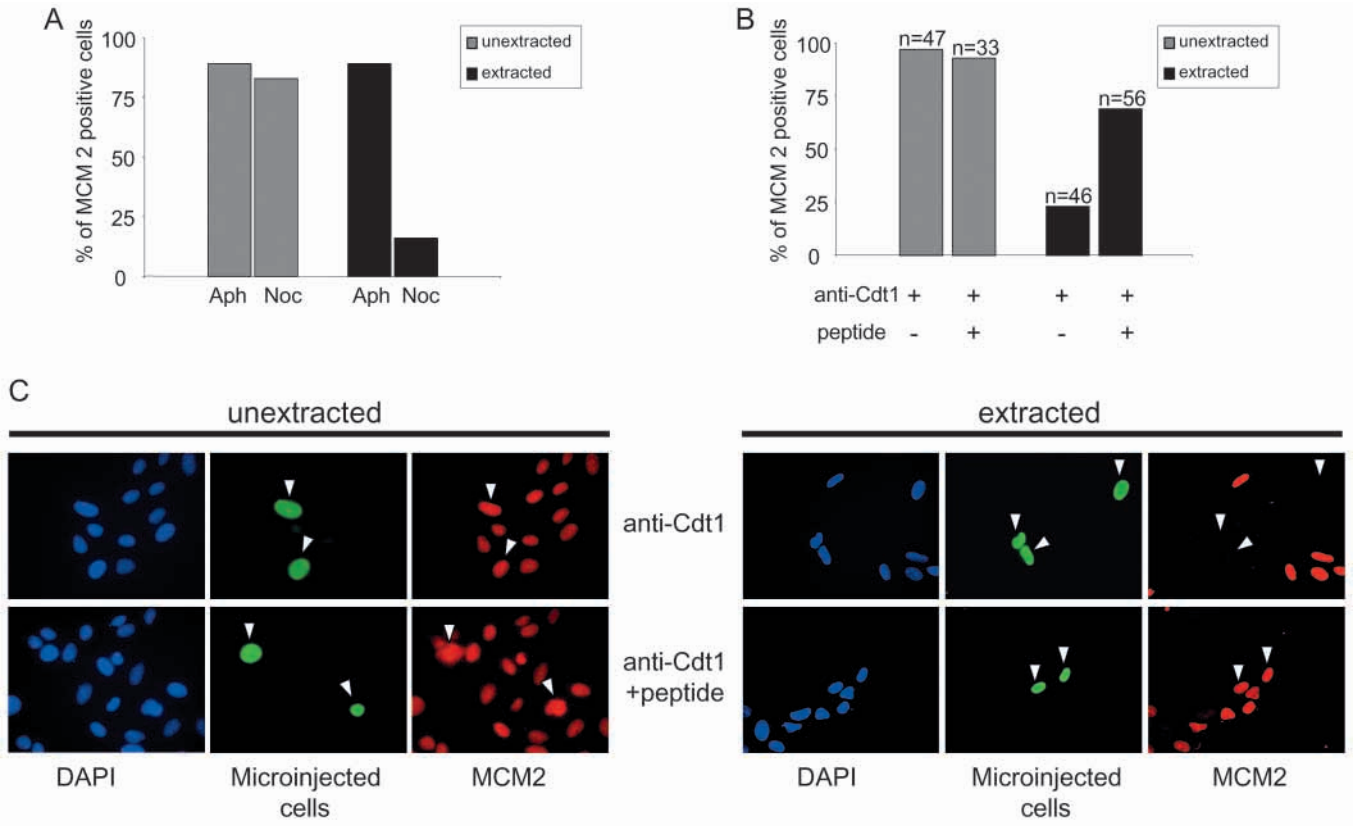

Fig. 4. hCdt1 is required for the loading of MCM2 onto chromatin. (A) U2OS cells were synchronized either at G1/S border with aphidicolin (5 μg/ml) for 24 hours or at G2/M transition with nocodazole (50 ng/ml) for 16 hours as indicated. The graph summarizes the number of cells showing MCM2 nuclear staining with (black bars) or without (grey bars) Triton X-100 extraction before fixation. (B) U2OS cells were treated as in Fig. 3A. The graph summarizes the percentage of cells showing MCM2 chromatin binding in the indicated conditions. Representative fields are shown in C. Original magnification ×550.

occurred and MCM proteins are tightly bound to chromatin (Krude et al., 1996; Aparicio et al., 1997). Moreover, in HU-treated cells, a number of early firing origins have already been activated and replication origin function is no longer required for the bulk of DNA synthesis (Vassilev and Russev, 1984; Bousset and Diffley, 1998; Santocanale and Diffley, 1998). As shown in Fig. 3B, 82% of cells microinjected with anti-Cdt1 antibodies and 80% of cells microinjected with control antibodies efficiently recover DNA replication after HU release. The pattern of BrdU staining was similarly uniform in microinjected and control cells. These data, together with the finding that the same reagents fully block DNA synthesis when microinjected into mitotic cells indicate that hCdt1 executes its function for DNA replication sometime between mitosis exit and the G1/S boundary. Consistent with the observation from fission yeast (Nishitani et al., 2000) our results demonstrate that hCdt1 is not required for DNA replication elongation in human cells.

Cdt1 is necessary for chromatin licensing

Execution point experiments, expression pattern and genetic evidence from model organisms hinted that hCdt1-dependent inhibition of DNA synthesis might occur by preventing chromatin licensing. This can be monitored as the binding of MCM proteins to DNA; in particular MCM2 protein, a subunit of the MCM complex, is always nuclear but its binding to DNA is cell cycle regulated. Treatment with low concentrations of non-ionic detergents discriminates between a chromatin engaged and a free MCM2 form (Krude et al., 1996; Okuno et al., 2001). We confirmed this finding since 83% of cells blocked in G1/S showed a Triton-resistant staining for MCM2, while only 16% of cells blocked in early mitosis showed a similar staining pattern (Fig. 4A). We coupled this assay to the microinjection approach to determine the requirement of hCdt1 for licensing. Mitotic U2OS cells were microinjected with anti-Cdt1 antibodies plus or minus blocking peptide, allowed to go through G1 and trapped at the G1/S border with aphidicolin in order to avoid loss of MCM2 binding due to completion of DNA replication. Microinjection of anti-Cdt1 antibodies, plus or minus competing peptide, did not change the normal nuclear MCM2 staining (Fig. 4B, grey bars). However, when a Triton wash was performed before fixation, inhibition of hCdt1 function caused MCM2 to be released from the nucleus in 77% of injected cells. MCM2 chromatin binding was almost fully rescued when anti-Cdt1 antibodies were pre-incubated with antigen peptide (Fig. 4B, black bars).

Discussion

The temporal separation between formation of pre-RCs and their activation is the invariable strategy that eukaryotic

organisms have developed to ensure that origins of replication are activated only once per cell cycle. Although the architecture of proteins involved in such a process appears to be conserved in different organisms, important differences exist in their regulation, most probably reflecting the intrinsic differences of cell cycle machinery (Diffley, 2001). Therefore it is important to extend the information provided by model organisms to the human system.

This work provides direct evidence that human Cdt1 is required for DNA replication in intact cells. The striking correlation between the number of microinjected cells incapable of replicating their DNA and the number of cells that do not load MCMs argues that hCdt1 executes its function in DNA replication by licensing the chromatin. Further studies will be necessary to understand how hCdt1 cooperates with the ORC complex and Cdc6 in building a licensed human origin. Our results indicate that, in the human cell cycle, hCdt1 function is required before the duplication of DNA. In highly proliferative tumor cells licensing occurs late in mitosis possibly at the anaphase/telophase transition (Mendez and Stillman, 2000; Okuno et al., 2001). Nonetheless, when normal cells exit the cell cycle to enter a quiescent state, some licensing proteins such as Cdc6 and MCM5 are actively degraded (Williams et al., 1998; Petersen et al., 2000; Stoeber et al., 2001); because of this active degradation in resting cells, their detection represents a valuable assay for identification of malignant cells in tissue samples (Williams et al., 1998; Stoeber et al., 1999). With respect to this, we observed that in serum-deprived cells hCdt1 protein is still detectable whereas Cdc6 is not present, suggesting that different mechanisms controlling the levels of these two licensing factors may exist. The presence of hCdt1 protein in G0 and early G1 cells is also consistent with the notion that addition of Cdc6 protein is sufficient per se to cause premature MCM loading and entry into S-phase of G1 nuclei in a mammalian cell-free system (Stoeber et al., 1998). Differences in the regulation of Cdc6 and Cdt1 proteins in cycling cells were recently described by Nishitani and co-workers (Nishitani et al., 2001). In particular, they have shown that in S-phase hCdt1 is unstable and targeted for proteolysis whereas in the same period of the cell cycle most of the Cdc6 protein is inactivated by a CDK2-dependent nuclear export mechanism (Petersen et al., 1999). CDKs play a crucial role in regulating licensing activity. Intriguingly, de-regulation of CDK activity causes genomic instability both in model organisms and in cancer cells (Brown et al., 1991; Spruck et al., 1999; Noton and Diffley, 2000). In budding yeast, this correlates with a lower efficiency of origin usage (Palmer et al., 1990; Hogan and Koshland, 1992; Noton and Diffley, 2000). Ultimately it will be important to define how cyclin-dependent kinases affect the activity of each component of the licensing system, including hCdt1, in normal human cells, and whether genomic instability observed in some cancers might be caused solely by impairment of pre-RCs formation and defective origin usage.

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