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Cdt1 and geminin: role during cell cycle progression and DNA damage in higher eukaryotes

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1. ABSTRACT

DNA replication in eukaryotic cells must be strictly regulated to ensure that the entire genome is duplicated only once in each cell cycle. For this purpose, the initiation of DNA replication is controlled by the "licensing" reaction, which is established by the formation of a pre-replicative complex (pre-RC) with the sequential assembly of the origin recognition complex (ORC), Cdc6, Cdt1 and Mcm2-7 onto origin regions. Among these, Cdt1 is likely the most important target for regulating licensing in higher eukaryotic cells, since illegitimate accumulation of Cdt1 causes multiple rounds of DNA replication without an intervening mitosis. Cdt1 is regulated over the course of the cell cycle mainly by the controlled expression of an inhibitor protein, geminin, and the level of Cdt1 periodically fluctuates due to ubiquitination and proteolysis. While the expression of geminin from S phase to metaphase of mitosis prevents licensing, Cdt1 accumulates from M to G1 phases and is degraded at the onset of S phase. Furthermore, Cdt1 is also proteolyzed in G1 phase in response to DNA damage, presumably providing a new checkpoint control.

2. INTRODUCTION

The gene encoding Cdt1 was originally identified in *Schizosaccharomyces pombe* by isolating genomic DNA sequences bound by the *cdc10* gene product (1), a transcription factor necessary for the completion of START. The transcription of *cdt1* depends on a functional *cdc10* product and is tightly regulated in a cell cycle-dependent manner, with transcript levels peaking significantly in G1 phase. Also, *cdt1* is an essential gene and disruption results in defects in DNA replication (1).

The *cdc18* gene product, a fission yeast ortholog of the Cdc6 protein in *Saccharomyces cerevisiae* and higher eukaryotes, has a central role in regulating S phase onset, as overproduction bypasses a control that prevent DNA from being replicated more than once per cell cycle, resulting in continual DNA synthesis without nuclear or cell division (2). In a screening to identify proteins that cooperate with Cdc18, Cdt1 was found to enhance the over-replication phenotype of cells overexpressing Cdc18, although overexpression of Cdt1 alone did not significantly affect DNA content (3, 4).

Orthologs of Cdt1 have been also found in human (5, 6), mouse (7, 8), *Xenopus laevis* (9), *Drosophila melanogaster* (10), *Caenorhabditis elegans* (11) and *Saccharomyces cerevisiae* (12, 13), suggesting a conserved function in regulating eukaryotic DNA replication. In addition, there is evidence for a Cdt1 inhibitor, geminin, in higher eukaryotic cells (5, 14-17), but not in *S. pombe* or *S. cerevisiae*. This finding predicts that Cdt1 plays an important role in regulating the initiation of DNA replication in higher eukaryotes, in which DNA synthesis must be more strictly controlled than that in yeast. Indeed, numerous reports have demonstrated or suggested that re-replication can be induced by enhancing Cdt1 activity in higher eukaryotic DNA replication (11, 18-25), whereas Cdc6 appears to have a supporting function with respect to Cdt1 overexpression (19). This review summarizes the findings concerning the function and the regulations of Cdt1 and geminin involved in higher eukaryotic DNA replication.

3. FUNCTIONS OF CDT1 IN EUKARYOTIC DNA REPLICATION

3.1. Cdt1 as a licensing factor for DNA replication

One of first implications of Cdt1 in the initiation of DNA replication in higher eukaryotes was provided by analysis of a cell-free replication system based on *Xenopus* egg extracts (9). The gene encoding *Xenopus* Cdt1 was originally isolated from maternally enriched RNA in a search for genes expressed early in embryos. A requirement for Cdt1 in replication was suggested by the finding that depletion of Cdt1 from *Xenopus* egg extracts results in a replication deficiency, although nuclear assembly is unaffected.

Subsequently, *Xenopus* Cdt1 was found to have an activity that was previously attributed to an element termed replication licensing factor B (RLF-B) (15). The idea of "licensing" for DNA replication was originally proposed by Blow and Laskey, who predicted the existence of replication licensing factors (26). In their original hypothesis, the licensing factor was assumed to be associated with chromatin and to be required for the initiation of replication. Thus, the factor operates as if it issues a "license" to the chromatin for replication in S phase. In addition, it was also proposed that the factor dissociates from chromatin or is inactivated as replication progresses, and that re-activation of the factor is prevented until nuclear envelope break down in mitosis. These features of a licensing factor should ensure that replication occurs only once per cell cycle.

Analysis of the replication licensing factor revealed that its activity is provided by two components, RLF-M and RLF-B (27). The RLF-M fraction contains the Mcm2-7 complex (27), which was first identified in *S. cerevisiae* as a group of minichromosome maintenance proteins that play a role in plasmid replication (28). The Mcm2-7 complex associates on chromatin before the initiation of replication and dissociates during the elongation phase (27, 29-32), thus fulfilling at least some of the prerequisites for a licensing factor.

Several lines of evidence strongly suggested that the Mcm2-7 complex is a DNA helicase that resolves the DNA duplex ahead of the replication machinery (33). Mcm2-7 proteins possess motifs conserved in DNA/RNA helicases (34) and form a hexameric ring structure typical of many DNA helicases (35, 36). A complex composed of Mcm4, Mcm6 and Mcm7 indeed exhibits DNA helicase activity *in vitro* (37). In addition, experiments with *Xenopus* egg extracts suggested that Cdc45 and CDK activities activated DNA helicase activity in the Mcm2-7 complex (38), and that Mcm2-7 promotes chromosome unwinding during replication (39, 40). Thus, the licensing for replication is thought to entail the loading of the replicative DNA helicase onto an origin region, although activation of the helicase is not included in this process (41).

On the other hand, RLF-B, which was subsequently revealed to be Cdt1, seems to function in loading the Mcm2-7 complex onto chromatin prior to DNA replication, although it is distinct from the origin recognition complex (ORC) or Cdc6 (42), both of which must bind chromatin before Mcm2-7 is loaded (43, 44).

3.2. Steps in the formation of the pre-replicative complex

Currently, it is understood that replication licensing is established by the formation at replication origins of a protein-DNA complexes, which is termed the pre-replicative complexes (pre-RCs) (45, 46) (Figure 1). Pre-RC formation is initiated by the binding of ORC onto chromatin. This complex, discovered as a factor that binds *S. cerevisiae* origins of replication in an ATP-dependent manner (47), is composed of six subunits including three AAA+ ATPases (48, 49). Although replication origins in higher eukaryotes are less defined, ORC has been conserved during evolution and is required for the initiation of DNA replication in different species (50).

The loading of Cdc6 onto chromatin, which depends on the prior association of ORC with origins, is also required for the recruitment of Mcm2-7 (43). Conversely, Cdc6 appears to modulate binding selectivity and stability of ORC (51, 52). Similarly, artificial targeting of Cdc6 to specific DNA regions promotes local ORC assembly and the initiation of replication in an ORC-dependent manner (53). These findings suggest that Cdc6 modifies ORC function at replication origins. Cdt1 has also been demonstrated to bind to chromatin, which depends on the prior association of ORC (20, 54). Although Cdc6 and Cdt1 independently bind chromatin (20, 54), it has been suggested that Cdt1 must be recruited after Cdc6 to permit the Mcm2-7 to be loaded (55).

The assembly of ORC, Cdc6 and Cdt1 provides competence to load Mcm2-7 onto origin regions (41, 56, 57), and pre-RC formation, or licensing for replication, is completed with the inclusion of this complex. Although the precise molecular mechanism by which Mcm2-7 is loaded is not well understood, it has been shown that purified ORC, Cdc6, Cdt1 and Mcm2-7, in addition to

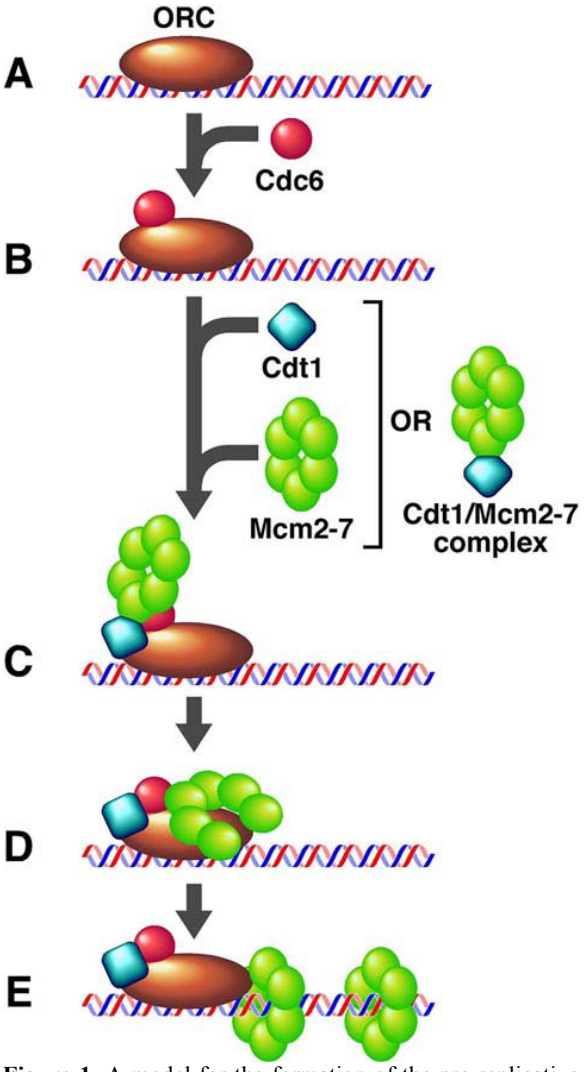

Figure 1. A model for the formation of the pre-replicative complex (pre-RC). The ATP-bound form of Cdc6 is loaded onto ORC associated with or near the replication origin (A-B). Cdt1 then binds chromatin, and Mcm2-7 gains access to chromatin in a Cdt1-dependent manner. It is also possible that Cdt1 associates with Mcm2-7 prior to chromatin loading and that it recruits the latter complex to the origin region (B-C). Cdt1 conveys Mcm2-7 to the Cdc6-ORC complex (C-D), and Cdc6-ORC opens the Mcm2-7 ring to allow it to be loaded onto double-stranded DNA with accompanying ATP hydrolysis by Cdc6 (D-E). Formation of the pre-RC is completed with the loading of multiple copies of Mcm2-7 onto a single origin region (E).

nucleoplasmin which mediates a structural transition of sperm chromatin, promote origin licensing in demembranated *Xenopus* sperm nuclei (58), suggesting that only these factors are essential for licensing. Recent findings also implicate Mcm8, a protein related to Mcm2-7 (59, 60), in pre-RC formation in human cells (61), whereas depletion of Mcm8 from *Xenopus* egg extracts reduces replication activity without affecting pre-RC formation (62). In any case, it is likely that Mcm8 is an auxiliary rather than essential factor for replication.

As described above, Mcm2-7 loaded *de novo* onto chromatin is thought to be inactive, and activation, which involves CDK and Cdc7-Dbf4 kinase activities, must occur before initiation of replication. In the last decade, the activities and proteins involved in the transition from the pre-RC to the initiation have been extensively studied: these findings are reviewed elsewhere (41, 57, 63, 64).

Once replication has been licensed, the affinity of ORC and Cdc6 for chromatin is significantly weakened and they become sensitive to CDK activity (65-67), suggesting that their mode of association is somehow changed by the presence of Mcm2-7. It has been suggested that the association of Cdt1 with chromatin is also destabilized by Mcm2-7 loading (58). In addition, the licensed chromatin lacking ORC, Cdc6 or Cdt1 is still replicated in ORC-, Cdc6- or Cdt1-depleted *Xenopus* egg extracts (54, 66, 67). Thus, it appears that Cdt1, like ORC and Cdc6, is no longer required for initiation after licensing is established.

3.3. A possible Cdt1 role in the loading of Mcm2-7 onto chromatin

Taking these observations together, replication licensing is achieved by the sequential assembly of ORC, Cdc6, Cdt1 and the Mcm2-7 complex (Figure 1). This scenario implies that only Cdt1 plays a direct role in recruiting Mcm2-7 to chromatin, and that ORC and Cdc6 merely provide a "landing pad" for Cdt1. This idea, however, may not fully explain licensing. Cdc6 shares structural similarity with the ORC subunits, Orc1 (68, 69) and thus is another AAA+ ATPase required for pre-RC formation (49). It has been reported that the binding of ATP to Cdc6 is required to load it onto chromatin, while hydrolysis of the bound ATP is necessary for loading Mcm2-7 in *Xenopus* egg extracts (58, 70). ATPase activity of *S. cerevisiae* Cdc6 has also been demonstrated to participate in loading Mcm2-7 onto yeast origins (71-73). Thus, Cdc6 must actively participate in recruiting Mcm2-7.

The importance of ATP with respect to the role of ORC in loading Mcm2-7 has been most extensively examined in yeast. The origin-specific binding of ORC depends on the binding of ATP by Orc1 (74). ORC containing ATP-bound Orc1 supports the initial loading of Mcm2-7 without ATP hydrolysis (75), whereas this initial Mcm2-7 loading requires ATP hydrolysis on Cdc6 (73). On the other hand, repeated rounds of Mcm2-7 loading onto a single origin region require hydrolysis of ATP by ORC (75). This reiterative loading of Mcm2-7 is also likely to occur in higher eukaryotes, as 10 to 40 copies of Mcm2-7 have been estimated to bind to chromatin for each ORC in *Xenopus* egg extracts (76, 77).

Since Mcm2-7 form a ring shaped complex similar to that of proliferating cell nuclear antigen (PCNA), a sliding clamp for DNA polymerase, there may be a loader of the complex onto duplex DNA similar to replication factor C (RF-C), which is specific for PCNA loading (78, 79). RF-C is composed of five AAA+ ATPase proteins similar to Cdc6, Orc1, Orc4 and Orc5. Moreover, sequence and structural similarities have been identified for RF-C subunits and Cdc6 (71, 80). Therefore, a model for Mcm2-

7 loading onto chromatin can be extrapolated from the role of RF-C in PCNA loading; here, the ORC-Cdc6 complex transiently opens the Mcm2-7 ring to allow it to engage duplex DNA in an ATP-dependent manner (57).

What role does Cdt1 have in this process? Recombinant Cdt1 co-precipitates with the Mcm4/6/7 complex expressed in a baculovirus system or with the Mcm2/4/6/7 complex purified from *Xenopus* egg extracts, possibly through a direct interaction with Mcm6 (8, 81). Subunits of the Mcm2-7 complex were also found to co-precipitated with exogenous or endogenous Cdt1 from nuclease-treated crude cell extracts (82, 83), suggesting that Cdt1 interacts with the Mcm2-7 complex under physiological conditions. Furthermore, Cdc6 has been shown to interact with Cdt1 in mammalian and fission yeast cells (3, 82). Taking these observations into account, it has been speculated that Cdt1 associates with Mcm2-7 before its chromatin loading and brings the complex to the origin region in order to introduce Mcm2-7 to the ORC-Cdc6 complex situated at the origin. Indeed, it has been suggested that the Cdt1 function must be provided after the chromatin loading of Cdc6, although Cdt1 and Cdc6 independently associates with chromatin (55). On the other hand, experiments using *Xenopus* egg extracts have demonstrated that Mcm2-7 can be loaded onto chromatin after the recruitment of Cdt1 (9, 55). These experiments, however, were made under non-physiological conditions in which Cdt1 and Mcm2-7 were not simultaneously provided. Thus, these results indicate that Cdt1 can function in Mcm2-7 loading after it associates with chromatin, but they do not necessarily reflect the physiological behavior of Cdt1 or exclude the possibility that Cdt1 associates with Mcm2-7 prior to binding chromatin.

3.4. Biochemical and structural properties of Cdt1

Thus far Cdt1 has been shown to have affinities for geminin, Orc2, Mcm6, Cdc6, cyclin A, PCNA, and DNA, but it has no demonstrated enzymatic activities. In addition, the primary structure of Cdt1 lacks motifs associated with enzymatic activities. These observations further suggest that Cdt1 is an adaptor protein that bridges the Mcm2-7 and Cdc6-ORC complexes.

Cdt1 is divided into two functional regions, a N-terminal regulatory region and a functional domain that includes the middle and C-terminal part of the molecule, which is sufficient for licensing in *Xenopus* egg extracts (81). Although the N-terminal region is poorly conserved, it contains a PCNA-binding motif (PIP-box) (84, 85), a cyclin-binding consensus motif (Cy-motif) (86, 87) and a phosphorylation site possibly recognized by CDK (21). These motifs are required for Cdt1 proteolysis (see below). It has been also demonstrated that the regulatory region weakly contributes to geminin binding (88).

The principal interface for interaction with geminin is located in the middle part of the Cdt1 (8, 81, 83), while the domain that binds Mcm4/6/7, Mcm2/4/6/7 or Mcm6 alone is located in the C-terminal region (8, 81). Geminin likely inhibits the interaction between Cdt1 and Mcm2-7 by steric hindrance rather than by direct competition for a binding domain (83). It has been indicated that the N-terminal part of Cdc6 interacts with Cdt1 (82), and the middle part of Cdt1 appears to be responsible for the interaction (T. Tsuyama and S. Tada, unpublished observation). The region that interacts with Orc2 has not yet been identified.

Cdt1 binds single- and double-stranded naked DNA in a sequence- and conformation-independent manner (8). It has been speculated that the N-terminal and middle regions of Cdt1 independently contribute to DNA-binding (8). The interaction between DNA and Cdt1 is inhibited by geminin or by CDK phosphorylation (8, 87), although the functional relevance of DNA binding has not yet been established.

4. GEMININ

The licensing system must be strictly regulated to achieve once-per-cell-cycle replication. As mentioned above, Cdc18/Cdc6, rather than Cdt1, appears to be important for regulation in *S. pombe*. In *S. cerevisiae*, re-replication in G2 phase can be induced by a combination of mutations that have a Cdc6 variant resistant to proteolysis, ORC subunits lacking consensus sites for CDK phosphorylation, and Mcm2-7 constitutively localized to the nucleus (89). On the other hand, Cdt1 appears to be a key regulatory target in higher eukaryotic cells, because numerous systems influence its activity during the cell cycle and inappropriate accumulation of the activity results in higher ploidy levels caused by re-replication. One of the higher eukaryotic regulatory mechanisms lacking in yeast cells is the inhibition of Cdt1 activity by geminin.

4.1. Geminin as an endogenous inhibitor of the licensing reaction

Geminin is a nuclear protein of relatively low molecular weight that was discovered in a screen for substrates of the *Xenopus* anaphase promoting complex/cyclosome (APC/C) (14). It was originally suggested that geminin has a regulatory function in the licensing reaction, and it was subsequently found that it specifically binds and inhibits Cdt1 in human cells and *Xenopus* egg extracts (5, 15). The tight association of geminin with Cdt1 prevents the latter protein from interacting with Mcm2-7 (8, 82, 83). In addition, it has been indicated that geminin also suppresses the binding of Cdt1 to DNA or to Cdc6 (8, 82). Thus, once Cdt1 associates with geminin, its functions with respect to licensing appears to be inhibited in multiple ways, although Cdt1 still interacts with chromatin even when it is bound by geminin.

As indicated by the enhancement of Cdt1 activity, re-replication of DNA is observed in human and *Drosophila* cells depleted of geminin activity (16, 90-92). Significant levels of re-replication coupled with re-licensing also result when Cdt1 is supplemented to *Xenopus* egg extracts depleted of geminin (22, 23). These results support the ideas that geminin plays a crucial role in regulating Cdt1 function and that the balance between Cdt1

and geminin activities is important for the maintenance of genomic integrity (93).

4.2. Geminin domains required for Cdt1 inhibition

Amino acid sequence analysis revealed that geminin has a mitotic cyclin-like destruction box in its N-terminal region and five heptad amino acid repeats predicted to form a coiled-coil in the middle of the molecule (14, 83, 88). Geminin is mitotically degraded in an APC/C- and destruction box-dependent manner (14, 94), while the minimal essential region for the inhibition of the licensing is situated in the middle region of the protein that includes the coiled-coil domain (14). Two clusters of basic amino acids between the destruction box and the coiled-coil region form a bipartite nuclear localization signal (23, 95). This domain, in addition to the destruction box and the sequence between the two regions, is required for the degradation of geminin (95).

Meanwhile, geminin was independently isolated as a protein that induces neural development in *Xenopus* embryos (96). This finding suggests that geminin has two distinct activities. Indeed, the neuralizing activity was assigned to its N-terminal region, which is separable from the minimal essential domain for the inhibition of DNA replication (14, 96).

Two geminin molecules are self-associated via their coiled-coil domains to form a dimer (83, 88, 95), or possibly tetramer as suggested by crosslinking experiments (97). This dimerization is critically required for the association with and inhibition of Cdt1 (88, 95). Crystallography analysis revealed that the N-terminus of the coiled-coil and its adjacent regions directly associate with the middle or N-terminal part of Cdt1 after dimerization (83, 88). On the other hand, the C-terminal stretch of the coiled-coil region of geminin is thought to sterically hinder the interaction between Cdt1 and Mcm2-7 and this region is thus critical for inhibiting Cdt1 activity (83).

4.3. Regulation of geminin in the cell cycle

4.3.1. The early embryonic cell cycle

Geminin is suggested to inhibit licensing from S phase to metaphase in *Xenopus* egg extracts (15, 98). Once metaphase-arrested *Xenopus* eggs are released into interphase, geminin appears to be inactivated because the licensing process is activated (76, 99). Significant proportion of molecules, however, seems to escape degradation at the metaphase-anaphase transition in *Xenopus* egg extracts (98), and the Cdt1-inhibiting activity of these remaining molecules must be suppressed other than by proteolysis. Indeed, the association of geminin with Cdt1 varies over the cell cycle in *Xenopus* egg extracts: association is significant in metaphase extracts but not in interphase extracts containing residual geminin (98). In addition, externally added geminin is efficiently degraded upon exit from metaphase even in egg extracts (14, 98), suggesting that some particular pool of geminin escapes the APC/C-mediated degradation.

Externally added geminin associates with and inhibits Cdt1 in interphase egg extracts (14, 15, 100), indicating that modification of geminin, rather than that of Cdt1 or transition of circumstantial conditions, most likely explains the inability of geminin to inhibit Cdt1 in interphase extracts. Further analysis has demonstrated that the activation of licensing, or the inactivation of geminin, depends on the geminin destruction box and APC/C activity but not on proteolysis (100). Therefore, it has been suggested that geminin is inactivated by ubiquitination and that the inactivated state is sustained, although ubiquitination is transient and de-ubiquitinated geminin escapes proteolysis (100).

The subpopulation of geminin that is inactivated probably mediates the inhibition of licensing during the following S phase. The remaining geminin in interphase extracts can acquire the ability to inhibit licensing and to associate with Cdt1 after these extracts are incubated with *Xenopus* sperm nuclei (98). It has also been suggested that re-activation depends on nuclear formation and import of the surviving geminin into the nucleus (98), although the detailed mechanism of the inactivation and re-activation of geminin has yet to be clarified.

4.3.2. The somatic cell cycle

In contrast to what is observed in egg extracts, geminin rapidly disappears at the end of mitosis and accumulates during S phase in cultured cells (5, 6, 14). This discrepancy may relate to differences between the embryonic and somatic cell cycles, rather than to species-specific differences, because geminin also completely disappears at anaphase in cultured cells derived from *Xenopus* (14). The control of geminin protein level over the cell cycle is achieved by proteolysis from late mitosis to G1 and by transcription of geminin mRNA (94, 101), although the geminin mRNA level varies only two- to three-fold over the cell cycle, with a peak at the G1/S transition (6, 101). The transcription of geminin is reportedly under the control of several E2F family transcription factors, which is inhibited by retinoblastoma tumor suppressor (102, 103).

Approximately half of the geminin expressed in S phase associates with chromatin even in the absence of Cdt1, possibly as a precaution to prevent Cdt1 from gaining access to chromatin in case that it accidentally accumulates during S-phase (101). Geminin was also shown to be phosphorylated in a cell cycle-dependent manner, with the extent of phosphorylation increasing during S-phase (104), although the physiological relevance of this modification remains to be clarified.

4.4. Other possible functions for geminin in the cell cycle

In addition to its inhibition of Cdt1, it has been suggested that geminin is also involved elsewhere during the cell cycle. With respect to Cdt1, geminin has also been proposed to have a role in the positive regulation of the licensing reaction. Depletion of geminin in human cells by siRNA technique destabilizes Cdt1 and impairs following round of DNA replication (105). Similarly, Cdt1 is rapidly down-regulated after geminin is silenced in *Drosophila* cells (91). It is suggested that the binding of geminin to Cdt1 protects the latter protein from ubiquitination and

subsequent proteolysis (105). This effect may be relevant to the findings that geminin, as well as Cdt1, is highly expressed in tumor cells and is down-regulated upon cell cycle exit into the G0 state (106-108).

When geminin is depleted from developing *Xenopus* embryos by treatment with antisense oligonucleotides, cells arrest in G2 phase immediately after the midblastrula transition following normal early embryonic cell cycles (109). Cell cycle arrest is caused by abrogation of Cdc2 activation through the Chk1-mediated checkpoint pathway (109). Although it has been reported that depletion of geminin results in re-replication that induces Chk1 activation, which in turn prevents further replication (90-92), the region of geminin essential for inhibiting replication does not appear to completely overlap with the region that prevents G2-specific arrest in *Xenopus* embryos (95). Thus, geminin may have a direct role in suppressing the checkpoint response. In this regard, it is interesting that geminin was identified in a search for proteins that associate with human Chk2 (91).

A possible function of geminin in centrosome duplication has also been proposed. Although this semi-conservative process occurs only once per cell cycle, over-duplication of centrosomes was detected in human cells depleted of geminin by the siRNA technique (110). The finding suggests that geminin is involved in the regulation of once-per-cell-cycle duplication not only for the genome but also for centrosomes. However, it is still possible that this effect is an indirect consequence of re-replication and subsequent activation of a checkpoint that arrests the cell cycle, because the prolongation of G2 phase can cause aberrations in centrosome number (111).

5. REGULATION OF CDT1 BY PROTEOLYSIS

Suppression of Cdt1 activity by geminin is one of the critical regulatory mechanisms to enforce the once-per-cell-cycle replication rule. However, depletion of geminin does not induce re-replication in *Xenopus* egg extracts (14, 25), suggesting that an additional mechanism also contributes to preventing re-replication. On the other hand, significant re-replication is found in geminin-depleted *Xenopus* egg extracts when Cdt1 is supplemented to the extracts after a first round of DNA replication, or when extracts are treated with the proteasome inhibitor MG132 (22, 23, 25). Moreover, in human cells, the overall profile of Cdt1 presents a striking contrast to that of geminin because Cdt1 accumulates during M and G1 phases, and rapidly disappears after the onset of S phase despite a relatively constant level of *cdt1* mRNA throughout the cell cycle (6, 105). These observations suggest the importance of the proteolytic control of Cdt1 in the regulation of licensing activity.

5.1. A CDK-dependent pathway mediated by SCF^{Skp2} ubiquitin ligase

Several reports have suggested that proteolysis of Cdt1 in S phase is mediated through poly-ubiquitination by SCF^{Skp2} ubiquitin ligase. Cdt1 physically interacts with components of the SCF^{Skp2} complex in human cells (112). Poly-ubiquitination of Cdt1 was observed *in vitro* using purified SCF^{Skp2} complex, and it is stimulated by overexpression of Skp2 (112), suggesting that SCF^{Skp2} contributes to the proteolysis of Cdt1.

Phosphorylation of Cdt1 by the cyclin-CDK complex is required for interaction between Cdt1 and Skp2 (86, 87). This modification depends on the cyclin-binding consensus motif (Cy-motif) located in the N-terminal region of Cdt1, which is readily recognized by cyclin A but not by mitotic cyclin B (87). In addition to the Cy-motif, a threonine residue located closer to the N-terminus of Cdt1 was identified as a critical phosphorylation site for the recognition by Skp2 (21). This finding suggests that the pathway for the proteolysis of Cdt1 is regulated by the activation of S phase CDK to suppress the licensing activity during DNA replication.

Mutation of the Cy-motif of Cdt1 impairs its association with Skp2 as well as with the cyclin-CDK complex (86, 87). Although the mutant protein is remarkably stable (86, 87), it does not escape degradation, whereas an N-terminally truncated version of Cdt1 appears to be completely stable (20, 21). This result indicates that there is an alternative pathway for Cdt1 proteolysis. Ectopic expression of a Cdt1 variant that does not function as a CDK-substrate, however, induces re-replication without notable overproduction of the mutant protein (21), suggesting the importance of SCF^{Skp2}-mediated proteolysis or phosphorylation by CDK in the regulation of Cdt1 function.

5.2. A PCNA-dependent proteolysis pathway

It has been suggested that Cdt1 is ubiquitinated and degraded in a manner dependent on DNA synthesis during DNA replication in *Xenopus* egg extracts (25). This mode of degradation seems to occur after Cdt1 associates with chromatin, which is not a consequence of pre-RC assembly but rather reflects some other interaction with chromatin (25, 84). A recent report indicates that the chromatin-dependent degradation of Cdt1 is due to its association with PCNA through a consensus PCNA-binding motif, or PIP-box, by the extreme N-terminus of Cdt1 (84). The degradation of Cdt1 is significantly suppressed by mutation of its PIP-box or by competitive inhibition of the association between Cdt1 and PCNA by the PIP-box of p21, suggesting that association with PCNA is required for Cdt1 proteolysis (84). As PCNA is a replication protein that accumulates on replication forks during S phase, efficient Cdt1 proteolysis triggered by its association with PCNA on chromatin should provide an additional mechanism to ensure that licensing is abrogated after the initiation of replication.

Similar observations have also been made in human cells (85, 113). Deletion of N-terminal region of human Cdt1 containing part of the PIP-box or mutations in the consensus amino acids in the PIP-box stabilizes the protein in S-phase when it is coupled with the mutation conferring a CDK-phosphorylation defect. On the other hand, neither mutation in the PIP-box nor the one affecting CDK-phosphorylation is sufficient to prevent Cdt1

degradation during S phase, suggesting that both the PCNA- and CDK-dependent pathways independently contribute to Cdt1 proteolysis (85, 113). Recently, it has been suggested that Cdt1 mutated in the Cy-motif is degraded in S-phase but not in G2-phase cells, whereas PIP-box mutant is degraded both S- and G2-phase cells (113). Thus, it is likely that these two proteolysis pathways are coordinately involved in Cdt1 degradation, in which PCNA-dependent proteolysis of Cdt1 operates only during DNA replication, while SCF^{Skp2} mediates ubiquitination and proteolysis of Cdt1 through S- and G2-phases.

It has been speculated that the $Cul4^{Ddb1}$ ubiquitin ligase complex is concerned with the PCNA-dependent proteolysis in S phase. The $Cul4^{Ddb1}$ ubiquitin ligase has been implicated in Cdt1 degradation following DNA damage in G1-phase (see below), a process in which PCNA is also involved (85, 114). In addition, it was demonstrated that an *S. pombe* mutant lacking the *ddb1* gene accumulates Cdt1 in the absence of DNA damage (114). In *Xenopus* egg extracts, Ddb1 interacts with Cdt1 and depletion of Ddb1 causes a significant reduction in Cdt1 ubiquitination during replication (84). Furthermore, suppression of *C. elegans* Cul4 by the RNAi method results in a striking increase in genome content, up to 100 C DNA in contrast to the 2C DNA content of wild type cells. This phenotype is coupled to the persistence of Cdt1 in S phase (11). These results suggest that the Cdt1 ubiquitination pathway controlled by $Cul4^{Ddb1}$ is conserved during evolution.

It has been found that the *Xenopus* CDK inhibitor $p27^{Xic1}$ is degraded in a PCNA-dependent manner (115), and putative human and *Drosophila* counterparts of $p27^{Xic1}$, $p27^{Kip1}$ and Dacapo, are stabilized by inactivation of Cul4 (116). Thus, it is likely that the PCNA- and Cul4-dependent regulation of proteolysis is not a unique mechanism for Cdt1 degradation.

5.3. Proteolysis of Cdt1 in response to DNA damage

Cdt1 is rapidly degraded upon UV or gamma irradiation without affecting the geminin level, possibly providing one of DNA damage checkpoint pathways to delay entry into S phase. This effect has been reported in human, *Drosophila*, and fission yeast cells (114, 117-119), suggesting that this mechanism is evolutionarily conserved. The degradation that occurs after gamma-irradiation is not mediated by activation of ATM, ATR, Chk1 or Chk2, which are involved in well-characterized DNA damage checkpoint mechanisms (117, 118), while the UV-induced degradation of Cdt1 has been attributed to ATR activation (118).

The degradation of Cdt1 after gamma or UV irradiation is dependent on Cul4, or both Cul4A and Cul4B in the case of human cells (117, 119). Cul4 is a member of cullin family proteins that associate with RING-finger E3 ubiquitin ligases (120). Deletion of the Cul4 ortholog from *S. pombe* results in elongated cells with decondensed chromosomes (121). Fission yeast Cul4 is implicated in heterochromatin assembly and ribonucleotide reductase regulation (122-125). In higher eukaryotes, suppression of Cul4 causes embryonic lethality, as seen in the mouse (126), and stabilization of CDK inhibitors, as seen in *Drosophila* and human cells (116). Cul4 has also been implicated in cyclin E regulation (116). Thus, Cul4 appears to be involved in numerous regulatory mechanisms in the cell cycle.

Cdt1 associates with Cul4 in human and *Drosophila* cells, and the extent of association increases after gamma or UV irradiation (117, 119). In addition, Cdt1 is polyubiquitinated by Cul4, as shown in a cell-free ubiquitination system consisting of Cdt1 and Cul4 isolated from irradiated or control cells (117, 119). The association between Cdt1 and Cul4 and the ubiquitibnation of Cdt1 depend on damaged-DNA binding protein 1, or Ddb1, which presumably bridges Cdt1 and Cul4 (119). Ddb1 is well known to interact with Ddb2 to form a damaged-DNA binding protein complex that tightly binds UV-irradiated DNA prior to nucleotide excision repair (127). Recent evidences suggests that Ddb1 functions in Cul4-mediated ubiquitination in cooperation with the COP9 signalosome (CSN) (120, 124, 125, 127), a multiprotein complex associated with variety of cullins (128-130). Indeed, the dependency of Cdt1 proteolysis on CSN subunits was also demonstrated after gamma irradiation of human cells (117). Thus, it is likely that Cdt1 is degraded immediately after DNA damage through a ubiquitination pathway involving Cul4 and CSN, in which Ddb1 functions in substrate recognition.

The UV-induced proteolysis of Cdt1 depends on its N-terminal region with the PIP-box, and reduction of PCNA stabilizes Cdt1 after UV irradiation (85, 114). Whereas interaction between Cdt1 and PCNA is almost undetectable in *Xenopus* egg extracts (84), this interaction is observed in human cell lysates (85, 114) and appears to be enhanced after UV irradiation of the cells treated with a proteasome inhibitor (85). These results suggest that the UV induced proteolysis of Cdt1 depends on the association between Cdt1 and PCNA, as speculated for $Cul4^{Ddb1}$-dependent proteolysis in S phase. Since PCNA is involved in multiple mechanisms including DNA replication and repair, it may control Cdt1 stability in further situations of a coordination of various metabolic pathways in the cell cycle.

While degradation of Cdt1 in S phase is likely to be mediated by SCF^{Skp2} ubiquitin ligase in addition to $Cul4^{Ddb1}$, it has been suggested that $Cul4^{Ddb1}$-dependent proteolysis is the sole pathway for the degradation of Cdt1 in response to UV-irradiation (85, 113). In contrast, it has also been reported that Skp2 contributes to the UV-induced degradation of Cdt1 (118). Moreover, although several observations suggest that the degradation pathway is somehow regulated by post-translational modification of Cdt1, possibly by phosphorylation, since the modified form of Cdt1 preferentially associates with Cul4, Ddb1 or Skp2 (117-119), this possibility has not been extensively examined. These issues, as well as the biochemical function of PCNA in degradation, should be clarified in the future.

6. SUPPRESSION OF LICENSING BY MITOTIC CDK

Protein level of Cdt1 reduced in S-phase is recovered during M-phase (6, 105) although the licensing

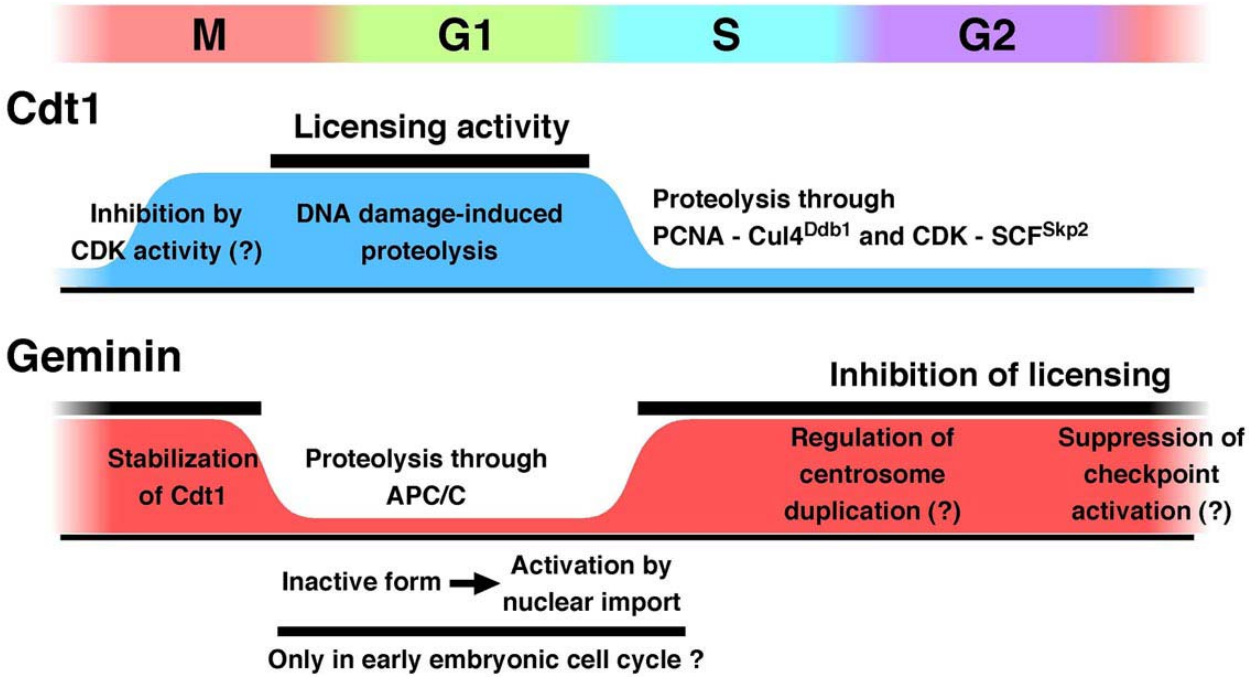

Figure 2. Regulation of Cdt1 and geminin protein levels in the cell cycle. The regulatory mechanisms and possible functions of Cdt1 and geminin described in the text are summarized here.

activity is suppressed until transition of metaphase and anaphase. Mitotic Cdk activity, in addition to geminin, appears to contribute to the prevention of the premature licensing without activating proteolysis of Cdt1 (15, 76). While mitotic inhibition of licensing by geminin is dominant over the contribution of mitotic CDK in *Xenopus* egg extracts (15), inactivation of CDK, but not silencing of geminin, has shown to result in premature licensing in M-phase of human cells (105). It has been demonstrated in metaphase-arrested extracts from *Xenopus* eggs that associations of ORC and Cdc6 towards chromatin are significantly enhanced by an inhibition of CDK activity (15), suggesting that the chromatin association of ORC, and possibly Cdc6 as well, is the target of the regulation. Moreover, it has been shown that Cdt1 is highly phosphorylated by mitotic CDK, and that suppression of this kinase activity leads to chromatin association of Cdt1 in M-phase of mammalian cells (87, 105). These results suggest that mitotic CDK negatively regulates Cdt1 activity. It is indicated that geminin less efficiently associates with unphosphorylated Cdt1 in M-phase cells treated with a CDK-inhibitor (105). Thus, the CDK-dependent inhibition of Cdt1 activity may be achieved by phosphorylation of Cdt1 that promotes its binding with geminin.

7. SUMMARY AND PERSPECTIVES

As described in this review, Cdt1 is a central target for the regulatory mechanisms that restricts DNA replication to once per cell cycle in higher eukaryotes. The regulation of Cdt1 is mainly achieved by an endogenous inhibitor, geminin, and by proteolysis during S phase, as summarized in Figure 2. Moreover, recent findings indicate that Cdt1 is rapidly proteolyzed after DNA damage, providing a possible new checkpoint control in G1 phase that delays entry into S phase. Considering that PCNA, which coordinates multiple cellular processes, is involved in the two pathways that lead to Cdt1 proteolysis, Cdt1 degradation may occur not only during the G1-S transition and in response to DNA damage, but also during numerous other cellular processes. Remarkable advances have been made in understanding this regulation, but the mechanistic details of Cdt1 function and the coordination of the activity with higher order cellular processes await further analysis. In addition, the MYST-family histone acetyltransferase Hbo1 was recently implicated in regulating the licensing reaction (131) and thereby may provide another control of Cdt1. These findings emphasize the possibility that there are other unidentified mechanisms that influence Cdt1 activity and its role in replication licensing.

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