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Licensing for DNA replication requires a strict sequential assembly of Cdc6 and Cdt1 onto chromatin in *Xenopus* egg extracts

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ABSTRACT

Replication origins are licensed for a single initiation event by the loading of Mcm2-7 proteins during late mitosis and G1. Sequential associations of origin recognition complex, Cdc6 and Mcm2-7 are essential for completion of the licensing. Although Cdt1 also binds to the chromatin when the licensing reaction takes place, whether the binding is a requirement for Cdt1 to function is unclear. To analyze the relevance of the chromatin association of Cdt1, we carried out chromatin transfer experiments using either immunodepleted *Xenopus* egg extracts or purified proteins. Licensing assay and immunoblotting analyses indicated that Cdt1 could only license DNA replication and load Mcm2-7 onto DNA when it binds to chromatin that has already associated with Cdc6. These results provide evidence supporting that Cdc6 and Cdt1 must bind to chromatin in a strict order for DNA licensing to occur.

INTRODUCTION

In the eukaryotic cell cycle, chromosomal DNA is precisely duplicated in S phase and segregated to daughter cells in M phase. The initiation of DNA replication is tightly regulated by the DNA replication licensing system so that it occurs only once per cell cycle. At an early stage of the cell cycle, a pre-replication complex (pre-RC) is formed and the chromatin becomes 'licensed' for DNA replication. Formation of the pre-RC is carried out by the loading of proteins such as the origin recognition complex (ORC), Cdc6, Cdt1 and the mini-chromosome maintenance (Mcm) protein complex onto a replication origin (1–4). First, ORC binds to the origin, then Cdc6 and Cdt1 attach to the chromatin in an ORC-dependent manner (5,6), and finally Mcm2-7 is loaded, which is, in turn, dependent on the binding of Cdc6 (7,8).

Mcm protein complex has been thought of as a replicative helicase and it was previously reported that Mcm4, 6 and 7 form a complex possessing helicase activity (9,10). At present, it is believed that the loading of Mcm2-7 is the entity of the licensing reaction for DNA replication. ORC, Cdc6 and Cdt1 are essential for the loading of Mcm2-7 onto chromatin. Cdc6 was originally identified as a protein essential for cell proliferation in yeast (11). The binding of Cdc6 to the chromatin at the early stage of the cell cycle has been shown to be essential for DNA replication in *Xenopus* egg extract and human cells (6,12). Cdc6 is a member of the AAA+ ATPase family and exhibits significant sequence similarity to the eukaryotic clamp loader, RF-C, and therefore, is thought to act as a clamp loader for the Mcm complex (13,14).

Cdt1 was originally found in *S.pombe* as a protein whose expression was induced by Cdc10, a CDK-dependent transcription factor (15). Later on, Cdt1 was found to possess an activity referred as replication licensing factor-B (RLF-B) that had previously been identified in *Xenopus* egg extract (16–18). Cdt1 also binds to chromatin as a component of pre-RC. The binding of Cdt1 is dependent on the association of ORC but not Cdc6 with the chromatin (5,19). More recently, an *S.cerevisiae* homolog of Cdt1 has been shown to be included in the pre-RC and to function in the loading of Mcm2-7 (20,21). This finding suggests that the licensing system is widely conserved in eukaryotes. Geminin, an inhibitor of the licensing system, was originally identified as a substrate of anaphase promoting complex (APC) (22). The protein was shown to bind to Cdt1 and to inhibit its activity for the loading of Mcm proteins (18,23). Accordingly, degradation of geminin via ubiquitination by APC leads to activation of Cdt1 and progression of the licensing reaction. As previously reported, loss of geminin induces a Cdt1-dependent re-replication and checkpoint kinase activation (24–26). Moreover, expression of Cdc6 and Cdt1 in G2 phase reportedly induced re-replication in fission yeast (27). Thus, the regulation of both Cdc6 and

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Cdt1 plays a crucial role in preventing re-replication until the next cell cycle. Furthermore, a recent report showed that Cdt1 was a critical and evolutionarily conserved proteolytic target of the DNA damage checkpoint in G1 phase. The CUL4/ROC1-associated ubiquitin ligase and COP9-signalosome have a unique function in the degradation of Cdt1 in response to DNA damage. Thus, proper regulation of Cdt1 activity may be important for preventing genomic instability and tumorigenesis (28,29).

It is thought that the proteins involved in the licensing reaction carry out their functions after they have associated on the chromatin, and this is quite likely the case for ORC, Cdc6 and Mcm2-7. Cdt1 is also loaded onto the chromatin before DNA replication is initiated. An *in vitro* study indicated that mouse Cdt1 bound to DNA directly in a sequence-, strand- and conformation-independent manner (30). However, there is no report addressing the relevance of the chromatin binding of Cdt1 to its function in the loading of Mcm2-7. In this study, we examined the functional significance of the chromatin binding of Cdt1 using a *Xenopus* egg extract cell-free system. We found that, although Cdt1 functioned in the loading of Mcm2-7 after associating with the chromatin, the preceding binding of Cdc6 to the chromatin was essential for the function of Cdt1. These results provide evidences for a new intermediate step in the licensing reaction and suggest that sequential assembly of Cdc6 and Cdt1 is required for the licensing of DNA replication.

MATERIALS AND METHODS

Preparation of *Xenopus* egg extract

Extracts derived from metaphase-arrested *Xenopus* eggs were prepared according to the method described by Chong *et al.* (31). The extract was used after a release to interphase by an addition of 0.3 mM $CaCl_2$ and 15 min incubation at 23°C. *Xenopus* sperm nuclei were prepared after demembranation with lysolecithin as described previously (31).

Production of recombinant proteins

Rabbit polyclonal Cdt1 antibody was raised against recombinant full-length *Xenopus* Cdt1 fused with a His6-tag (His-Cdt1). A plasmid expressing His-Cdt1 was generously provided by Drs D. Maiorano and M. Mechali (5). Rabbit polyclonal Cdc6 antibody was raised against His6-tagged full-length *Xenopus* Cdc6 expressed in BL21-Codon Plus RIL (Stratagene). His6-tagged-geminin was expressed from *Xenopus* geminin H cDNA lacking a destruction box and purified using Ni-NTA agarose (Qiagen). A plasmid expressing the recombinant protein was generously provided by Drs T. McGarry and M. Kirshner (22). *Xenopus* Cdt1 fused with glutathione-S-transferase (GST-Cdt1) was expressed in BL21-Codon Plus RIL and purified using Glutathione-Sepharose (Amersham Biosciences). GST-Cdt1, in contrast to His-Cdt1, was efficiently recovered in a soluble fraction of *E.coli* lysate, and thus was used as an active protein. The RLF-B activity of GST-Cdt1 was verified by measuring the ability to supplement licensing activity in a Cdt1-depleted *Xenopus* egg extract.

Purification from *Xenopus* egg extract

The egg extract for purification was prepared after *in vivo* activation of *Xenopus* eggs by an addition of A23187 as described previously (31). Fifty percent (w/v) polyethylene glycol 6000 (PEG) (Wako) in 20 mM HEPES-KOH, pH 8 was added to the extract to make 5% PEG to obtain precipitated and supernatant fractions. A partially purified fraction containing Cdc6 and Cdt1 (BPAS fraction) was obtained from the 5% PEG precipitated fraction by a process based on a method described previously (16,31,32). In brief, the precipitated fraction was solubilized in T'LFB1 (40 mM HEPES-KOH, pH 8.0, 20 mM potassium phosphate, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 10% (w/v) sucrose, 0.01% (v/v) Triton X-100, 1 μg/ml pepstatin and 1 μg/ml leupeptine) supplemented with 150 mM KCl, and adsorbed onto phosphocellulose resin (P11, Whatman). The adsorbed proteins were eluted with T'LFB1 containing 500 mM KCl. The BPAS fraction was obtained by 40% saturated ammonium sulfate precipitation from the phosphocellulose eluate and was solubilized in T'LFB1. A partially purified Cdc6 fraction was prepared from the BPAS fraction after removal of Cdt1 as indicated in 'Immunodepletion'.

Mcm2-7 complex was partially purified from the 5% PEG supernatant fraction. The fraction was subjected to further precipitation by 12% PEG, and the 5–12% PEG precipitated fraction was adsorbed by Q-Sepharose FF (Amersham Biosciences) in T'LFB1 containing 100 mM KCl. The adsorbed proteins were eluted with T'LFB1 containing 350 mM KCl, and precipitated with the addition of an equal volume of 50% PEG. The purified fraction was solubilized with T'LFB1 and stored at −80°C prior to use.

Isolation of chromatin fraction

For immunoblotting experiments, demembranated sperm nuclei were incubated with *Xenopus* egg extract at 23°C for a given period. The extract was diluted in 1 ml of nuclear isolation buffer (NIB; 50 mM HEPES-KOH pH 7.6, 50 mM KCl, 2 mM DTT, 2 mM $MgCl_2$, 0.5 mM spermidine, 0.15 mM spermine, 1 μg/ml pepstatin and 1 μg/ml leupeptin) supplemented with 0.1% Triton X-100 and 2.5 mM ATP (TNIBA), and 15% (w/v) sucrose in TNIBA (100 μl) was layered under the diluted extract. The chromatin fraction was precipitated at 9000 *g* in a swing bucket centrifuge for 5 min at 4°C. After a wash with 1 ml of TNIBA, the chromatin precipitate was subjected to SDS-10% polyacrylamide gel electrophoresis for immunoblotting.

For biochemical assays to detect licensing activity, chromatin after an incubation with the *Xenopus* egg extract and/or samples were diluted in 1 ml of NIB supplemented with 0.01% Triton X-100 and 2.5 mM ATP, and 100 μl of the same buffer containing 15% sucrose was layered under the diluted extract. The chromatin was then precipitated at 6000 *g* in a swing bucket centrifuge for 5 min at 4°C. The upper layer and supernatant of the lower layer were carefully removed until 20 μl of the buffer remained in the bottom of the tube. After resuspension by gentle pipetting, 2 μl of the isolated chromatin fraction was used for the licensing assay.

Immunodepletion

Immunodepletion was performed using anti-Cdt1 or anti-Cdc6 antibody incubated with protein A-Sepharose Fast Flow (Amersham Pharmacia), and washed with 100 mM HEPES-KOH, pH 8.0 for 1 h at room temperature. After the wash, the resultant Sepharose resin was incubated with 0.25 mM phenylmethylsulfonylfluoride for 15 min at room temperature, and thoroughly washed with LFB2 (40 mM HEPES-KOH, pH 8.0, 20 mM potassium phosphate, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 10% (w/v) sucrose, 2.5 mM ATP, 1 μg/ml pepstatin and 1 μg/ml leupeptine) supplemented with 50 mM KCl to obtain an antibody-coupled Sepharose. The *Xenopus* egg extract was incubated with the same volume of the antibody-coupled Sepharose for 1 h at 4°C. The extract after removal of the Sepharose resin was used as a depleted extract.

This protocol was repeated a second time for Cdc6-depletion. Although it was not necessary to repeat the process for Cdt1-depletion, the protocol was performed twice to prepare Cdt1-depleted extract when it was to be compared with Cdc6-depleted extract. Where the number of rounds of Cdt1-depletion needs to be clarified, we refer to the extract obtained after a single round of immunodepletion as 'Cdt1-single depleted extract' and that obtained after two rounds as 'Cdt1-double depleted extract'.

The removal of antigen proteins from protein fractions was also performed using the same depletion protocol. A Cdt1-free Cdc6 fraction was prepared by Cdt1-depletion from the BPAS fraction instead of the egg extract. It was verified that the fraction was able to complement the licensing activity in the Cdc6-depleted extract, but not in the Cdt1-depleted extract.

Measurement of DNA replication in Cdt1-depleted extract

Demembranated *Xenopus* sperm nuclei (100 000 nuclei) were incubated at 23°C for 20 min with 20 μl of depleted extract. After the incubation, the chromatin fraction was isolated from each extract and 2 μl of it was incubated at 23°C for 3 h with 10 μl of mock-treated or Cdt1-single depleted extract supplemented with 250 μg/ml cycloheximide, 25 mM phosphocreatine, 15 μg/ml creatine phosphokinase and [α-^{32}P]dATP (20 kBq). Total DNA synthesis during the second incubation was measured as the radioactivity incorporated into a fraction insoluble in 5% TCA as described previously (31,33). DNA synthesis is represented as a percentage of the radioactivity incorporated into DNA in the Cdt1-depleted extract to that in the mock-treated extract.

Licensing assay

To assay RLF-B activity in Cdt1-fractions, sperm nuclei (10 000 nuclei) were incubated for 20 min at 23°C with 2 μl of Cdt1-single depleted extract after a 2-fold dilution with LFB2 supplemented with 0.01% Triton X-100 (T'LFB2) containing the Cdt1-fractions to be tested. After the incubation, 10 μl of a fresh egg extract supplemented with 250 μg/ml cycloheximide, 25 mM phosphocreatine, 15 μg/ml creatine phosphokinase, 0.5 μM recombinant geminin and [α-^{32}P]dATP (20 kBq) was added to the reaction mixture. Geminin prevented further licensing, and therefore DNA replication during the following incubation period depended on the licensing activity in the first reaction mixture. Total DNA synthesized over a further 90 min incubation at 23°C was measured as the radioactivity incorporated into a fraction insoluble in 5% TCA as described previously (31,33).

For the assays indicated in Figures 2B and 5B, 15 μl of the chromatin fraction after the '1st incubation' was isolated and incubated for 20 min at 23°C with 30 μl of depleted extract or protein fraction for the '2nd incubation'. Then, 2 μl of the reaction mixture after the 1st or 2nd incubation was mixed with 10 μl of fresh egg extract supplemented with 250 μg/ml cycloheximide, 25 mM phosphocreatine, 15 μg/ml creatine phosphokinase, 0.5 μM recombinant geminin and [α-^{32}P]dATP (20 kBq) in the presence or absence of recombinant geminin (0.5 μM) without further isolation of the chromatin. DNA synthesis in the absence of geminin was taken as an index of the recovery of chromatin during the isolation process, and thus, licensing activity was given as a percentage of DNA synthesis in the geminin-containing extract to that in the geminin-free extract.

Elution of Cdt1 from chromatin

The chromatin fraction was isolated after the incubation of sperm nuclei (1 000 000 nuclei) with 200 μl of *Xenopus* egg extract for 20 min or 60 min at 23°C. Proteins bound on the chromatin were eluted by incubation of the isolated chromatin for 10 min on ice with 10 μl of T'LFB2 containing 300 mM KCl and 0.1% BSA. The eluted fraction was obtained as a supernatant after centrifugation at 9000 *g* for 10 min at 4°C, and stored at −80°C. The amount of Cdt1 in the eluted fraction was verified by immunoblotting analysis using known amounts of His-Cdt1 as authentic samples.

Immunoblotting

Samples for immunoblotting were electrophoresed on an SDS–10% polyacrylamide gel and electrically transferred onto a Hybond-P PVDF-membrane (Amersham Biosciences). The membrane was blocked with 10% skim milk in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.04 mM Na_2HPO_4 and 1.47 mM KH_2PO_4) and incubated with a primary antibody diluted 1000- to 3000-fold with PBS containing 0.05% Tween-20 and 3% BSA. After a wash with 0.05% Tween-20–PBS three times and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG, protein bands that reacted with the primary antibody were visualized with ECL western blotting detection reagents (Amersham Biosciences).

RESULTS

Relevance of chromatin binding of Cdt1

In order to assess the relevance of the association with chromatin of Cdt1 in DNA replication, sperm nuclei were incubated with mock-treated extract in the presence or absence of geminin, Cdt1-depleted extract, or Cdc6-depleted extract (Figure 1A). The chromatin fraction from each reaction mixture was incubated in Cdt1-depleted extract, and DNA synthesis during the incubation was measured (Figure 1B). While the licensed chromatin after the incubation with mock-treated extract was well replicated, chromatin was not replicated in the Cdt1-depleted extract when it was isolated

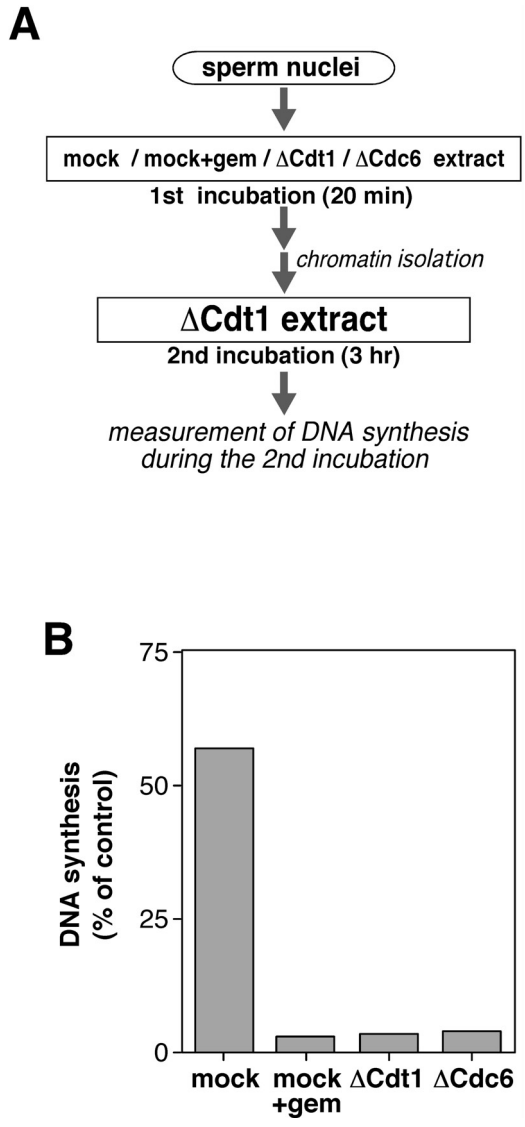

Figure 1. Cdc6-depleted chromatin is unable to replicate in Cdt1-depleted extract. (**A**) A schematic representation of an experimental procedure. *Xenopus* sperm nuclei (100 000 nuclei) were incubated for 20 min with 20 µl of Cdt1- or Cdc6-double depleted extract (ΔCdt1 or ΔCdc6, respectively), or mock-treated extract in the absence or presence of 0.5 µM recombinant geminin (mock or mock + gem, respectively). After the incubation, 2 µl of chromatin fraction isolated from each extract was incubated for 3 h with 10 µl of Cdt1-single depleted extract supplemented with [α-^{32}P]dATP (20 kBq). (**B**) Chromatin after the incubation with mock-treated extract without or with geminin (mock or mock + gem, respectively), Cdt1-depleted extract (ΔCdt1), or Cdc6-depleted extract (ΔCdc6) was isolated and further incubated with the Cdt1-single depleted extract. DNA synthesis during the second incubation is represented as a percentage of the radioactivity incorporated into DNA in the Cdt1-depleted extract to that in a mock-treated extract.

after the incubation with extract deficient in functional Cdt1 due to the addition of a Cdt1 inhibitor, geminin or immunodepletion of Cdt1. Surprisingly, the chromatin assembled in the Cdc6-depleted extract was also hardly replicated in the Cdt1-depleted extract despite that it should be associated with Cdt1 (see Figure 2C). The result suggests that the Cdt1 on the chromatin does not support the licensing for DNA replication in the Cdt1-depleted extract.

Licensing activity of chromatin-associated Cdt1

To confirm that the deficiency of DNA replication shown above was caused by a defect of licensing activity, we next examined the function of Cdt1 associated with chromatin in the licensing reaction. To this end, we prepared chromatin that was bound with all the proteins required for the licensing except Cdc6 or Mcm complex (Cdc6-depleted chromatin) by incubating sperm chromatin with a Cdc6-double depleted extract. Then, the chromatin was isolated and incubated with a Cdt1-double depleted extract. The occurrence of the licensing reaction during this sequential incubation was examined with a biochemical assay using a geminin-containing extract and the immunoblotting of chromatin-associated proteins (Figure 2). For a licensed control, we used mock-treated extract both for the first and for the second incubation. Even in this case, the loading onto chromatin of DNA polymerase α was not observed after the first or second incubation (data not shown), suggesting that nuclear formation or DNA replication did not occur during the first and second incubation possibly because of the dilution of the double depleted extracts.

We first confirmed that the immunodepletion removed only the targeted protein from the extract with little effect on the amounts of other proteins (data not shown). The licensing was assayed by measuring the amount of DNA synthesized after the addition of fresh egg extract supplemented with geminin, which prevents further licensing during the incubation (Figure 2B). Incubation of Cdc6-depleted chromatin with the Cdt1-depleted extract supplied Cdc6 to the chromatin, and therefore, the chromatin should have become licensed for DNA replication if the chromatin-bound Cdt1 on the Cdc6-depleted chromatin was active in the second incubation. Following this sequential incubation, however, very little or no synthesis of DNA in the licensing assay or loading of Mcm4 onto the Cdc6-depleted chromatin was observed in spite of the significant loading of Cdt1 and Cdc6 (Figure 2B and C). The results indicate that chromatin-bound Cdt1 does not function in the licensing process under these conditions.

Moreover, we prepared a chromatin fraction assembled in the Cdt1-depleted extract (Cdt1-depleted chromatin), and subsequently incubated the chromatin with the Cdc6-depleted extract so that the order of the incubations was reversed. A significant level of licensing activity and the loading of Mcm4 were observed after the second incubation in the Cdc6-depleted extract (Figure 2B and C), indicating that these depleted extracts were functional for the licensing except the function of the depleted proteins. The result also suggests that chromatin-associated Cdc6 was functional for the licensing when it was loaded onto chromatin in the absence of Cdt1.

The amount of Cdt1 associated with the chromatin was not a limiting factor for the licensing

The simplest explanation for the surprising result shown in Figures 1 and 2 is that the amount of Cdt1 associated with Cdc6-depleted chromatin was not large enough to exhibit significant RLF-B activity. To address this possibility, we quantified the amount using known quantities of His-Cdt1. We detected more than 0.7 pmol of Cdt1 on the chromatin of 80 000 nuclei incubated in the Cdc6-depleted extract (Figure 3A), that is, $\sim 5.3 \times 10^6$ molecules/nucleus.

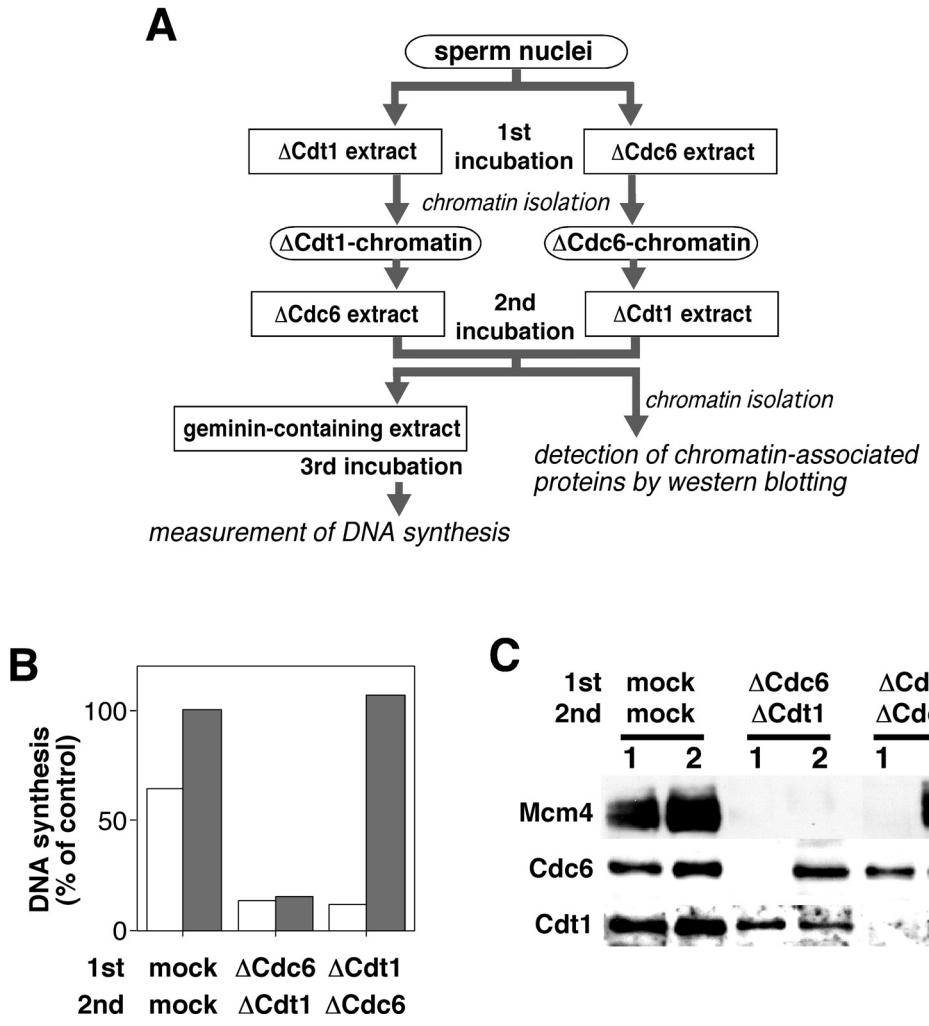

Figure 2. Cdc6-depleted chromatin is incapable of being licensed for DNA replication. (**A**) A schematic representation of experimental procedures. *Xenopus* sperm nuclei (100 000 nuclei) were incubated for 20 min with 20 µl of Cdt1- (ΔCdt1) or Cdc6-double depleted (ΔCdc6) extract (1st incubation), resulting in Cdt1-depleted chromatin (ΔCdt1-chromatin) or Cdc6-depleted chromatin (ΔCdc6-chromatin), respectively, after isolation of the chromatin fraction. The Cdt1-depleted or the Cdc6-depleted chromatin was further incubated for 20 min with 20 µl of Cdc6- (ΔCdc6) or Cdt1-double depleted (ΔCdt1) extract, respectively (2nd incubation). For a biochemical assay detecting the licensing activity, a fresh egg extract supplemented with 0.5 µM of recombinant geminin and [α-^{32}P]dATP (20 kBq) was added to the reaction mixture without an isolation of the chromatin and incubated for 90 min (3rd incubation). For an analysis of chromatin-bound proteins, the chromatin fraction was isolated from each reaction mixture of the 2nd incubation and subjected to immunoblotting. (**B**) The result of the biochemical assay for the licensing activity was indicated. Reaction mixtures after the 1st and 2nd incubation were added to a fresh egg extract in which the licensing activity was inhibited by geminin. DNA synthesis during a further 90 min incubation ('3rd incubation' in A) was measured and plotted as a percentage of the radioactivity incorporated into DNA during the incubation in the geminin-containing extract to that in a geminin-free extract. Open and shaded bars indicate the DNA synthesis in the geminin-containing extract after the 1st and 2nd incubation, respectively. (**C**) Isolated chromatin prepared after the sequential incubation in mock-treated (mock), Cdt1-double depleted (ΔCdt1) and/or Cdc6-double depleted (ΔCdc6) extract was immunoblotted using indicated antibodies. The chromatin fractions after the 1st and 2nd incubation were applied to lanes 1 and 2, respectively.

We next examined the licensing activity in 2 µl of Cdt1-depleted extract supplemented with purified GST-Cdt1 (Figure 3B). We detected significant licensing activity in the Cdt1-depleted extract supplemented with 30 nM (~3.6×10^6 molecules/nucleus) or even lower concentrations of GST-Cdt1 (Figure 3C). These results indicate that the amount of Cdt1 on the Cdc6-depleted chromatin is not a limitation for the subsequent licensing reaction.

Licensing activity of Cdt1 released from licensed chromatin

We next assessed whether chromatin-associated Cdt1 was qualitatively altered from the soluble form. Sperm nuclei were incubated for 20 min with *Xenopus* egg extract to allow Cdt1 to associate with the chromatin (Figure 4B). Then, proteins associated on the chromatin were released by treatment with a buffer containing 300 mM KCl. Cdt1 was efficiently eluted from the chromatin using this treatment (data not shown) (6). The eluted fraction was examined for the ability to complement the licensing activity in the Cdt1-depleted extract (Figure 4A). The result indicated that Cdt1 eluted from the chromatin was active, suggesting that Cdt1 was not irreversibly inactivated by binding to the chromatin (Figure 4C). In spite of the 5- to 6-fold reduction in activity compared with GST-Cdt1, 30 nM of Cdt1 (~3.6×10^6 molecules/nucleus) seemed enough to exhibit significant

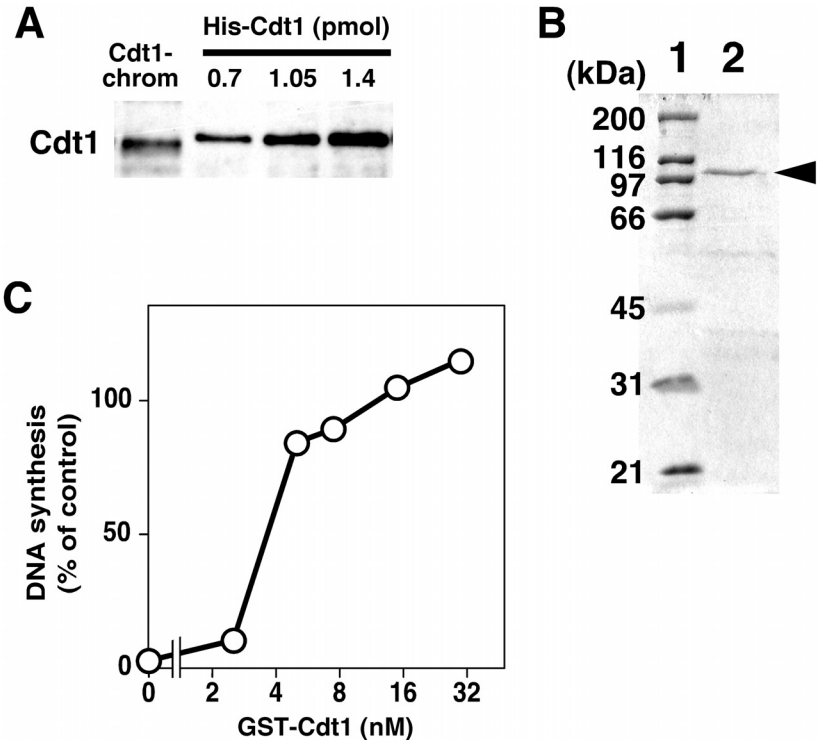

Figure 3. Cdc6-depleted chromatin associated with an amount of Cdt1 sufficient for RLF-B activity. (**A**) Sperm nuclei (80 000 nuclei) were incubated in 16 µl of Cdc6-depleted extract for 20 min and chromatin was isolated. Cdt1 on the isolated chromatin (ΔCdc6-chromatin) was detected by immunoblotting using anti-*Xenopus* Cdt1 antibody together with 0.7, 1.05 and 1.4 pmol of His-Cdt1. (**B**) GST-Cdt1 was electrophoresed and stained with Coomassie Brilliant Blue R-250. A molecular weight marker (lane 1) and 1.2 µg of GST-Cdt1 (lane 2) were applied to a SDS–10% polyacrylamide gel for electrophoresis. An arrowhead indicates the migrated position of GST-Cdt1. (**C**) Licensing activity was assayed with a Cdt1-depleted extract supplemented with GST-Cdt1. Sperm nuclei (10 000 nuclei) were incubated for 20 min at 23°C with 2 µl of Cdt1-single depleted extract after a 2-fold dilution with 0.01% Triton X-100 and 50 mM KCl in LFB2 containing increasing amounts of GST-Cdt1. The extent of the licensing reaction during the incubation was measured as the amount of DNA synthesized in a 3 h incubation with a geminin-containing extract supplemented with [α-^{32}P]dATP (20 kBq). The amount of DNA synthesized is represented as a percentage of the radioactivity incorporated into DNA during the incubation in the geminin-containing extract to that in a geminin-free extract.

licensing activity, which is lower than the amount of Cdt1 associated with the Cdc6-depleted chromatin estimated from Figure 3A (>5 × 10^6 molecules/nucleus).

To confirm whether the effect was dependent on Cdt1 in the eluted fraction, the fraction was immunodepleted of Cdt1 (Figure 4D). While the mock-treatment did not significantly affect the activity in the eluted fraction, very little or no licensing was observed when the eluted fraction depleted of Cdt1 was used. In addition, an eluted fraction from chromatin isolated after incubation for 60 min with egg extract was also incapable of supplementing the licensing activity in the Cdt1-depleted extract (Figure 4D). Cdt1 gradually dissociated from the chromatin during incubation with *Xenopus* egg extract in 30–50 min (Figure 4B) and was not detected on the chromatin isolated after incubation for 60 min (data not shown). Therefore, the result confirms that the licensing activity in Cdt1-depleted extract was restored by the addition of Cdt1 in the fraction eluted from the licensed chromatin. Considering this together with the result shown in Figure 3, it seems likely that the Cdt1 molecule associated with the chromatin is functional for licensing from both a quantitative and a qualitative point of view. These results suggest that the chromatin-based association of Cdt1 affects its function in the licensing system.

Chromatin-bound Cdt1 is functional only when it binds after Cdc6

Two possibilities were considered regarding the functional state of Cdt1. One was that there was a strict order to the binding of Cdc6 and Cdt1 onto chromatin for the loading of Mcm2-7. The other was that the Cdt1 molecule captured on the chromatin was no longer functional, and therefore, soluble Cdt1 in the reaction was necessary for the licensing process. To address these possibilities, we aimed to separate the loading of Mcm2-7 onto the chromatin from the loading of Cdc6 and Cdt1. To this end, soluble proteins were removed by the isolation of chromatin after the stepwise bindings of Cdc6 and Cdt1, and the resultant chromatin was incubated with the partially purified Mcm proteins (Figure 5).

GST-Cdt1 or partially purified Cdc6 was incubated with the Cdt1- or Cdc6-depleted chromatin, respectively. Then, the chromatin fraction was isolated and further incubated with the Mcm fraction. Cdc6 and Mcm were purified from egg extract to be separated from Cdt1 or each other's activity. Whereas the partially purified fractions were contaminated with other proteins, it was verified that neither the Cdc6 nor the Mcm fraction was active in the licensing reaction after the immunodepletion of Cdc6 and Mcm6, respectively (data not shown). The result indicated that Cdc6 or Mcm

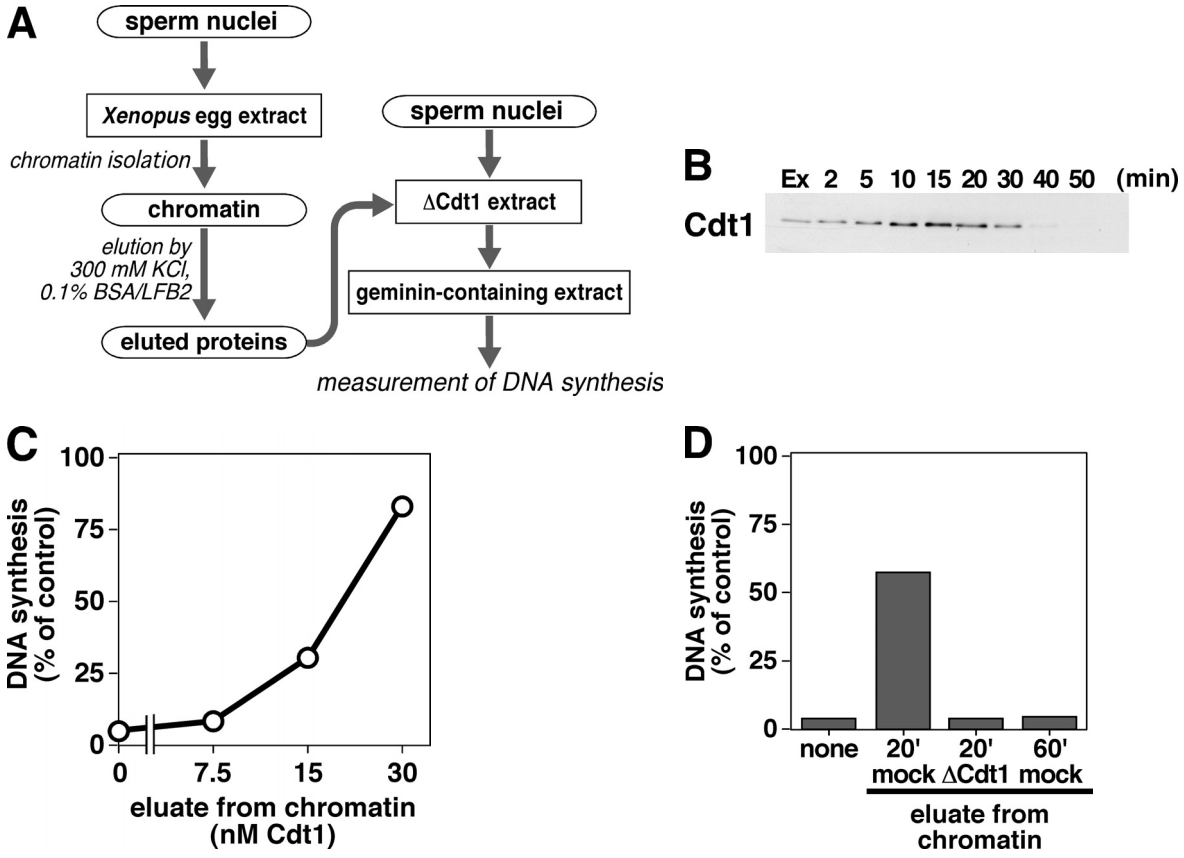

Figure 4. Cdt1 released from the licensed chromatin is active for the licensing. (**A**) The chromatin fraction was isolated after incubation of sperm nuclei (1 000 000 nuclei) with 200 µl of *Xenopus* egg extract for 20 min. Proteins bound on the chromatin were eluted by incubation of the isolated chromatin with a buffer containing 300 mM KCl and 0.1% BSA. Sperm nuclei (10 000 nuclei) were incubated for 20 min with 2 µl of Cdt1-single depleted extract after a 2-fold dilution with a buffer containing the elution fraction. After the incubation, the reaction mixture was further incubated for 90 min with 10 µl of a fresh egg extract supplemented with geminin (0.5 µM) and [α-^{32}P]dATP (20 kBq). The extent of DNA synthesis is represented as a percentage of the radioactivity incorporated into DNA during the incubation in the geminin-containing extract to that in a geminin-free extract. (**B**) *Xenopus* sperm nuclei were incubated for indicated periods in the interphase extract. After the incubation, the chromatin fraction was isolated from the extract and subjected to SDS–10% polyacrylamide gel electrophoresis. Cdt1 in the chromatin fractions was detected by immunoblotting. Two microliters of the interphase extract was applied onto the lane indicated by 'Ex'. (**C**) Proteins were eluted from chromatin after an incubation of sperm nuclei with egg extract for 20 min. Cdt1-single depleted extract was diluted 2-fold with a buffer containing the elution fraction corresponding to 0, 7.5, 15 or 30 nM Cdt1 and assayed for the licensing activity. (**D**) Proteins were eluted from chromatin after an incubation of sperm nuclei with egg extract for 20 or 60 min. Cdt1-depleted extract was diluted 2-fold with a buffer containing no eluate (none), an eluate from 20 min chromatin after a single depletion of Cdt1 (20' ΔCdt1) or after a mock treatment (20' mock), or an eluate from 60 min chromatin after mock treatment (60' mock).

proteins were only the active components of the partially purified Cdc6 or Mcm fraction, respectively.

As a control of the experiment, chromatin isolated from Cdt1- or Cdc6-depleted extract supplemented with GST-Cdt1 or the Cdc6 fraction, respectively, was used after mock treatments (Figure 5B, columns 1 and 8). The control chromatin was positive in the licensing assay, indicating that the sequential isolation did not disturb the detection of the licensed chromatin. When the Cdt1-depleted chromatin was incubated either with Cdt1 or with Mcm proteins, the licensed state was hardly detected (Figure 5B, columns 3 and 4). Similar results were obtained using the Cdc6-depleted chromatin after incubation with either Cdc6 or Mcm proteins (columns 10 and 11). On the other hand, a significant licensing reaction on the Cdt1-depleted chromatin was observed when Cdt1 and Mcm proteins were used for the first and second incubations, respectively (column 5). The extent of the licensing was equivalent to that of the control chromatin (columns 1 and 8), or the Cdt1-depleted chromatin after the incubation with a mixture of Cdt1 and Mcm proteins (columns 6 and 7). Whereas the serial incubation with Cdt1 and Mcm successfully licensed the Cdt1-depleted chromatin, no combination of Cdc6 and/or Mcm proteins was able to license the Cdc6-depleted chromatin (Figure 5B, columns 9–14). To confirm the results, proteins bound to the chromatin were identified by immunoblotting (Figure 5C). Although Cdt1 and Cdc6 were significantly associated with chromatin when they were supplied in the depleted extract or the first incubation mixture, Mcm4 was only detected on the chromatin prepared under the experimental conditions where licensing activity was detected in Figure 5B.

Although the results suggest that there is a strict order of association onto chromatin for Cdc6 and Cdt1 in order for licensing to occur, it is still possible that Cdt1 bound to chromatin was released during the second incubation, and that the released Cdt1, not the chromatin-associated Cdt1, functioned to recruit Mcm2-7 to the chromatin. Then, we next examined whether Cdt1 was released from the Cdc6 and Cdt1-associated

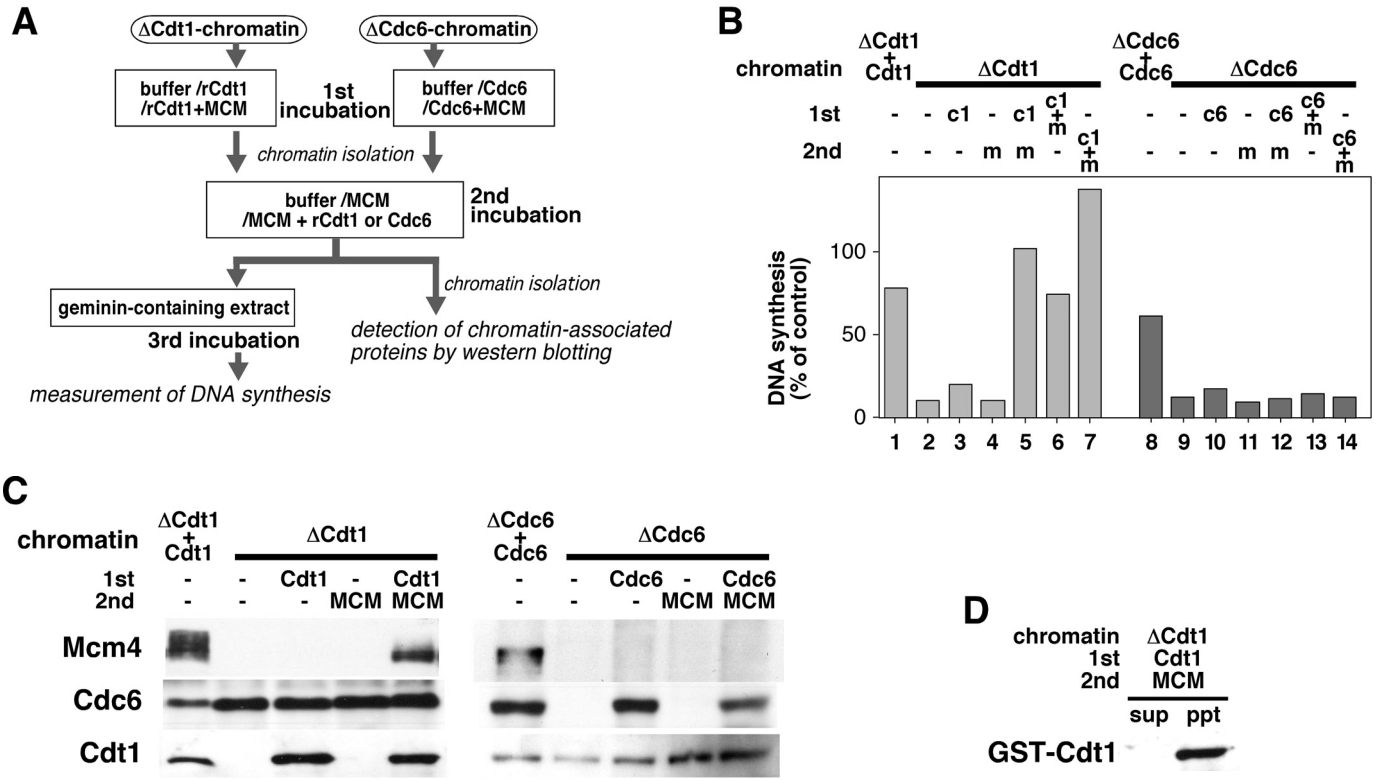

Figure 5. The stepwise association of Cdc6, Cdt1 and Mcm2-7 onto sperm nuclei. (**A**) A schematic representation of experimental procedures. Sperm nuclei (100 000 nuclei) were incubated in 20 μl of Cdt1- or Cdc6-double depleted extract for 20 min and then chromatin was isolated to prepare Cdt1-depleted chromatin (ΔCdt1 chromatin) or Cdc6-depleted chromatin (ΔCdc6 chromatin), respectively. The isolated chromatin was incubated for 20 min with GST-Cdt1, the Cdc6 fraction, a mixture of GST-Cdt1 and the Mcm fraction, or a mixture of Cdc6 and Mcm fractions (1st incubation). Chromatin was isolated again from the reaction mixture of the 1st incubation and incubated for 20 min with the Mcm fraction, a mixture of the Mcm fraction and GST-Cdt1, or a mixture of Mcm and Cdc6 fractions (2nd incubation). For a biochemical assay detecting the licensing activity, a fresh egg extract supplemented with 0.5 μM of recombinant geminin and [α-^{32}P]dATP (20 kBq) was added to the reaction mixture without an isolation of the chromatin fraction and incubated for 3 h (3rd incubation). For an analysis of chromatin-bound proteins, the chromatin fraction was isolated from each reaction mixture after the 2nd incubation and subjected to immunoblotting. (**B**) ΔCdt1- or ΔCdc6-chromatin was sequentially incubated with buffer (−), GST-Cdt1 (c1), Cdc6 fraction (c6) and/or Mcm fraction (m) as indicated. The samples were subjected to a biochemical assay to detect licensing activity. Isolated chromatin after an incubation with the Cdt1-depleted extracts supplemented with GST-Cdt1 (ΔCdt1 + Cdt1) and that after an incubation with the Cdc6-depleted extract supplemented with Cdc6 (ΔCdc6 + Cdc6) were used for controls of licensed chromatin (column 1 or 8, respectively). (**C**) The chromatin after the 2nd incubation was isolated and subjected to immunoblotting to detect Mcm4, Cdc6 and Cdt1. The bands of Cdt1 represent GST-Cdt1 in the left panel and native Cdt1 in the right panel. (**D**) Chromatin sequentially bound with Cdc6 and GST-Cdt1 was incubated with the Mcm fraction as described above. After the incubation with Mcm, the sample was separated into supernatant (sup) and chromatin (ppt) fractions by centrifugation. Both samples were immunoblotted with anti-Cdt1 antibody.

chromatin during the second incubation with the Mcm fraction (Figure 5D). As a result, no Cdt1 was observed in the supernatant fraction and almost all Cdt1 was detected in the precipitated fraction. This tight association of Cdt1 was also observed after the second incubation without the Mcm2-7 fraction, or after a shorter incubation resulting in an incomplete loading of Mcm2-7 (data not shown). The result further supports the notion that Cdt1 exerts licensing activity only when it binds to the chromatin after the loading of Cdc6. It should be also noted that Cdt1 seems to remain tightly associated on chromatin after the loading of Mcm2-7, whereas the affinity between Cdc6 and chromatin is significantly lowered after the Mcm2-7 loading (34).

Geminin does not influence the licensing activity of the chromatin-associated Cdt1

6-dimethylaminopurine (6DMAP) is known to prevent activation of the licensing system (35), and it inhibits Cdk-dependent activation of the APC that is necessary for the inactivation and degradation of geminin (36). Despite that the licensing reaction does not take place under these conditions, Cdt1 is detected in the chromatin fraction isolated from the 6DMAP-treated extract (5), and it is thought that chromatin-bound Cdt1 is associated and inhibited by geminin under such conditions (37).

The notion raises the possibility that the chromatin-associated Cdt1 is masked by geminin when the chromatin is assembled in the Cdc6-depleted extract. Indeed, geminin is normally found even in the interphase *Xenopus* egg extract although it appears completely inert (36,38). Then, it is possible that the interphase geminin is able to associate with Cdt1 and inhibits its activity after Cdt1 has been captured on the chromatin, despite that the interphase geminin cannot bind to soluble Cdt1. To examine this possibility, we carried out experiments similar to those shown in Figure 2 using an extract depleted of both Cdc6 and geminin, but obtained essentially the same results (Supplementary figure). Thus, it is unlikely

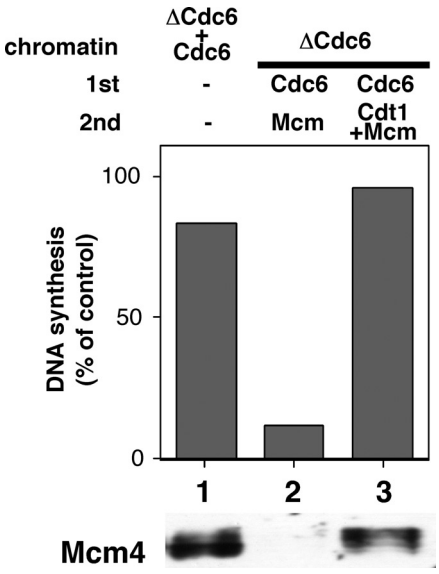

Figure 6. Cdc6 bound onto chromatin after Cdt1 functions in the licensing reaction. Sperm nuclei (200 000 nuclei) were incubated with 40 μl of Cdc6-depleted extract to isolate Cdc6-depleted chromatin. The Cdc6-depleted chromatin was incubated with the Cdc6 fraction for 20 min to allow the loading of Cdc6 onto the chromatin. The chromatin fraction was isolated and incubated with the Mcm fraction (column 2) or a mixture of the Mcm fraction and GST-Cdt1 (column 3) for 20 min. The chromatin isolated after the incubation with the Cdc6-depleted extract supplemented with Cdc6 (ΔCdc6 + Cdc6) was used as a control for licensed chromatin (column 1). A fresh egg extract (10 μl) supplemented with 0.5 μM of recombinant geminin and [α-^{32}P]dATP (20 kBq) was added to the reaction mixture of the 2nd incubation and incubated for 3 h to assess the licensing activity. The extent of DNA synthesis is represented as a percentage of the radioactivity incorporated into DNA during the incubation in the geminin-containing extract to that in a geminin-free extract (upper panel). The chromatin fractions after the second incubation were isolated and subjected to immunoblotting to detect Mcm4 (lower panel).

that the geminin remaining in the interphase extract inhibits the particular Cdt1 fraction associating with the chromatin.

Cdc6 bound to chromatin after Cdt1 association is functional for licensing

From the above experiments, we could not conclude which protein, Cdc6 or Cdt1, was defective in the function for licensing when the order of the association to chromatin was reversed. To resolve this issue, we examined whether Cdc6 bound to Cdc6-depleted chromatin was active for the licensing reaction when the chromatin was further incubated with Mcm proteins and Cdt1. An experiment similar to that shown in Figure 5B was performed by using Cdc6-depleted chromatin that associated with Cdt1. The Cdc6-depleted chromatin was incubated with the Cdc6 fraction to allow Cdc6 to associate on the chromatin. Then, the chromatin was isolated and incubated with a mixture of the Mcm fraction and GST-Cdt1 (Figure 6).

Consistent with the result in Figure 5B, a significant licensing reaction was detected when the Cdc6-depleted extract was supplemented with Cdc6 (column 1), and very little licensing was observed when the Cdc6-depleted chromatin was serially incubated with Cdc6 and Mcm (column 2). When GST-Cdt1 was supplied in the second incubation with the Mcm fraction, the licensing activity was comparable to that for the Cdc6-depleted extract supplemented with Cdc6. Immunoblotting analysis detecting Mcm4 supported the conclusion. The results indicate that Cdc6 loaded onto chromatin after Cdt1 is functional for the licensing reaction, suggesting that the function of Cdt1 is disrupted when it is loaded onto chromatin before Cdc6.

DISCUSSION

Relevance of the association of Cdt1 with chromatin to its function

Previous studies have indicated that the formation of pre-RC is accompanied by the association of Cdt1 with chromatin and the Cdt1 is then released with the activation of pre-RC (5,19). A biochemical analysis of Cdt1 revealed its ability to bind DNA directly in a sequence-, strand- and conformation-independent manner (30). In spite of these results, it remained unclear whether its association with chromatin is necessary for Cdt1 to function. It has been reported that Cdc6 and Cdt1 bind to chromatin independent of each other, although the binding of both proteins depends on the chromatin-associated ORC (5,19). It has already been revealed that Cdc6 functions on the chromatin and the process requires the hydrolysis of ATP (12,39). There is, however, limited biochemical information available on the function of Cdt1. In addition, the functional relationship between Cdc6 and Cdt1 is still unclear, even though it has been reported that they physically interact with each other in yeast and in mammalian cells (19,40). In this study, we have presented results suggesting a functional relationship between Cdt1 and Cdc6, which strictly depends on the order of their loading. Moreover, the result shown in Figure 5 indicates that Cdt1 functions after associating with the chromatin and the soluble form of Cdt1 is not required for the loading of Mcm2-7.

We have shown that chromatin associated with Cdt1, but not with Cdc6, cannot be replicated and licensed for DNA replication by incubation with Cdt1-depleted extract, which should provide Cdc6 and Mcm2-7. The result might mean that the chromatin-associated Cdt1 is not functional in quantitative or qualitative terms. Further analysis, however, reveals that the result is not accounted for by quantitative defects in the function of Cdt1 after its association with the chromatin. More than 5×10^6 molecules of Cdt1 are estimated to be loaded on a single nucleus, which is more than the number necessary for efficient licensing by recombinant Cdt1 or that recovered from the licensed chromatin.

The estimate for chromatin-associated Cdt1, 5×10^6 molecules per nucleus, seems very high (~20 molecules per origin) compared to that for ORC or Cdc6, which are suggested to provide a single or a few components for a single pre-RC formation (34,41). Although it has not been investigated whether all of these Cdt1 molecules are necessary for an efficient licensing reaction, it may mean that Cdt1 molecules associated on the chromatin were not only the ones to function in the licensing of origins on the recovered chromatin. This idea raises the possibility that most of the chromatin-associated Cdt1 molecules are non-functional, and little or no Cdt1 is functionally bound with the Cdc6-associated or the Cdc6-free chromatin, respectively. Thus, it will be

important to clarify differences between the functional and non-functional chromatin association of Cdt1 for a complete understanding of Cdt1's function on the chromatin. Whatever the status of the chromatin-binding of Cdt1 is, it is still possible to conclude that the amount of Cdt1 supplied from the Cdc6-depleted chromatin is more than that needed for significant licensing activity.

It was also possible that only soluble Cdt1 functions in the licensing of DNA replication. In such a case, the chromatin-associated (or captured) Cdt1 would be a form whose function was already finished, and thus, no longer active. To examine this possibility, we separated the loading of Mcm2-7 in the licensing process from the loading of Cdt1 or Cdc6 onto chromatin. The result indicated that chromatin-associated Cdt1 is active for Mcm2-7 loading when loaded after the chromatin-binding of Cdc6, but not active when loaded before the Cdc6 binding, supporting the notion that the order of the association of Cdc6 and Cdt1 is important for the licensing of DNA replication.

Probable significance of the strictly ordered binding of Cdc6 and Cdt1 to chromatin

Re-licensing must be avoided in the normal cell cycle, and there seems to be a number of mechanisms to ensure this. First, the licensing reaction is thought to occur in the absence of Cdk activity on which other cell cycle-related events, such as DNA replication and mitosis, are dependent (42–44). This difference in dependency on Cdk activity temporally separates the licensing reaction from the other cell cycle processes. Second, there is an endogenous Cdt1 inhibitor protein, geminin, in higher eukaryotic cells. The expression and degradation of geminin seems strictly regulated in the cell cycle, and geminin biochemically prevents uncontrolled activation of Cdt1 (23,38). Third, the amounts of Cdc6 and Cdt1 in the cells seem strictly controlled in the cell cycle. The mechanism limits the emergence of the licensing reaction during a small window of the cell cycle (27,45–47). In this study, we presented evidence of an order to the chromatin-binding of Cdc6 and Cdt1 for these proteins to function in the licensing system. This mechanism may also provide another regulatory mechanism to prevent the re-licensing of DNA.

It has been reported that the binding of ATP to Cdc6 is required for its binding with chromatin, and the hydrolysis of the bound ATP is necessary for loading of Mcm2-7 (37,39). Since Cdc6 is a member of the AAA+ superfamily and exhibits sequence similarity to RF-C, it is presumed to function like a clamp loader protein (13). However, there is no evidence of direct interaction between Cdc6 and Mcm complex, though accumulative evidence suggests that Cdt1 interacts with Cdc6 and Mcm complex (19,21,30,40). Therefore, Cdt1 may play a role in the linking of Cdc6 and Mcm complex in this process. Given that Cdc6 is an ATPase, it is interesting to know whether the assembly of Cdt1 onto pre-RC requires Cdc6-dependent hydrolysis of ATP. For this purpose, we have observed that Cdt1 bound to chromatin in the presence of ATPγS, a non-hydrolyzable analog of ATP. Moreover, the licensing reaction and Mcm4 loading were also detected after isolation of the chromatin from the ATPγS-containing reaction mixture followed by an incubation with ATP and the Mcm fraction (T. Tsuyama *et al*., unpublished observation). These results suggest that ATP hydrolysis catalyzed by Cdc6 is not required for the functional loading of Cdt1.

Why does the licensing reaction require a strictly ordered chromatin-binding by Cdc6 and Cdt1? We have shown that Cdc6 loaded on a chromatin that had already associated with Cdt1 appeared to function in the licensing reaction with Mcm2-7 and Cdt1 supplied after the Cdc6-loading. The result suggests that the activity of Cdt1, not Cdc6, was affected when the order of the binding was reversed. Recently, Cook and co-workers have shown that Cdc6 binds to Cdt1 at its N-terminal non-catalytic domain, and this interaction promotes the association of Cdt1 and Mcm complex (40). It is possible that the interaction between Cdt1 and Cdc6 only occurs when Cdc6 is associated with ORC prior to the binding of Cdt1.

In this paper, we provide evidence that the DNA replication licensing system requires a strict order of chromatin binding of Cdc6 and Cdt1, in which Cdc6 must bind to chromatin prior to the association of Cdt1 for the licensing reaction to occur. The phenomenon is evidence for cooperation between Cdc6 and Cdt1 for the loading of Mcm2-7 onto chromatin during the licensing process. Elucidating the mechanism of cooperation between Cdc6 and Cdt1 would provide a clue to the molecular basis of the licensing system.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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