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Functional domains of the *Xenopus* replication licensing factor Cdt1

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ABSTRACT

During late mitosis and early G1, replication origins are licensed for subsequent replication by loading heterohexamers of the mini-chromosome maintenance proteins (Mcm2-7). To prevent re-replication of DNA, the licensing system is down-regulated at other cell cycle stages. A small protein called geminin plays an important role in this down-regulation by binding and inhibiting the Cdt1 component of the licensing system. We examine here the organization of *Xenopus* Cdt1, delimiting regions of Cdt1 required for licensing and regions required for geminin interaction. The C-terminal 377 residues of Cdt1 are required for licensing and the extreme C-terminus contains a domain that interacts with an Mcm(2,4,6,7) complex. Two regions of Cdt1 interact with geminin: one at the N-terminus, and one in the centre of the protein. Only the central region binds geminin tightly enough to successfully compete with full-length Cdt1 for geminin binding. This interaction requires a predicted coiled-coil domain that is conserved amongst metazoan Cdt1 homologues. Geminin forms a homodimer, with each dimer binding one molecule of Cdt1. Separation of the domains necessary for licensing activity from domains required for a strong interaction with geminin generated a construct, whose licensing activity was partially insensitive to geminin inhibition.

INTRODUCTION

The metazoan genomes are of such considerable size that thousands of replication origins must be used in order for them to be replicated in a reasonable length of time. The use of these replication origins must be strictly regulated to avoid the over- or under-replication of any given region of the genome. In order to ensure that each replication origin initiates DNA replication no more than once per cell cycle, it must first be 'licensed' for replication before entering S phase (1,2). Licensing involves several different proteins, the origin recognition complex (ORC), Cdc6 and Cdt1 (3), and results in heterohexamers of Mcm2-7 being loaded onto the DNA. Mcm2-7 is essential for the initiation and elongation of replication forks, probably playing a major role in unwinding the DNA ahead of each replication fork. After entry into S phase, the activity of the licensing system is down-regulated to ensure that no replicated origin can become re-licensed, thus preventing origins firing more than once in each cell cycle.

Cdt1 plays an essential role in the licensing reaction (4–10). It is recruited to chromatin by ORC (3,4) and directly interacts with Mcm2-7 (10–12). The activity of Cdt1 is under strict cell cycle control, being negatively regulated by a small protein called geminin, which can bind tightly to Cdt1 (7,13–16). Geminin contains a lengthy section predicted to form a coiled-coil and which is required to inhibit Cdt1 (13). Re-replication of chromosomal DNA during S phase and G2 is prevented largely due to down-regulation of Cdt1 activity (17–21).

In this paper, we make a preliminary investigation of the structure of *Xenopus* Cdt1, determining regions required to provide licensing activity, and regions required for interaction with geminin. We also demonstrate that geminin forms a dimer and interacts with Cdt1 in a 2:1 molar ratio. While this work was in preparation, a paper was published showing a crystal structure of geminin complexed with a fragment of Cdt1 (22). Our results provide biochemical corroboration of some of the conclusions of this crystallographic work.

MATERIALS AND METHODS

Preparation and use of egg extracts

Metaphase-arrested *Xenopus* egg extracts were prepared as described previously (23). All extracts were supplemented with 250 μg/ml cycloheximide, 25 mM phosphocreatine,

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10 μg/ml creatine phosphokinase and 0.3 mM $CaCl_2$ before use, and incubations were performed at 23°C. *Xenopus* sperm nuclei were demembranated with lysolecithin, as described previously (23), and stored frozen at −80°C. For DNA synthesis experiments, sperm nuclei were incubated at a final concentration of 3 ng DNA/μl extract (~1000 nuclei/μl), and extracts (typically 10 μl) were supplemented with 50 μCi/ml [α-^{32}P]dATP. After a 90 min incubation, DNA synthesis was assessed by TCA precipitation, as described previously (23).

Immunodepletion of Cdt1 from extract was performed essentially as described previously (23). Anti-Cdt1 antibody was coupled to protein A–agarose beads at 2 ml antiserum per ml beads and washed in EDB-S buffer (50 mM HEPES at pH 7.6, 50 mM KCl, 2 mM DTT, 0.4 mM $MgCl_2$, 0.4 mM EGTA, 10% sucrose, and 10 μg/ml each of leupeptin, pepstatin and aprotinin). Metaphase-arrested extract was activated with 0.3 mM $CaCl_2$ and supplemented with 250 μg/ml cycloheximide, then mixed with 0.4 vol of antibody beads for 1 h at 4°C, after which the beads were removed. The process was repeated one more time. Immunoprecipitations were carried out using beads prepared using the above method.

Plasmids and cloning

Regions of *Xenopus* Cdt1 were expressed as glutathione *S*-transferase (GST) fusions in pGEX-2T (Pharmacia) encompassing the residues outlined in Figure 1. All constructs were cloned as BamHI–EcoRI restriction fragments through the introduction of restriction sites during PCR amplification (primer sequences available on request). Production of His-tagged Cdt1(193–447) and Cdt1(243–620) was carried out by recloning the insert from the relevant pGEX-2T clone into the appropriate pET28 vector.

To produce the Cdt1(193–447) plus mini-geminin complex, the pET-Duet1 vector (Invitrogen) was used. Cdt1(193–447) was cloned into MCS1 of pET-Duet1 as a BamHI–EcoRI fragment to contain an N-terminal His tag. Mini-geminin (residues 87–168) was cloned into MCS2 as a BglII–KpnI restriction fragment to contain a C-terminal S-tag (primer sequences available on request).

Recombinant protein production

Expression plasmids were transformed into *Escherichia coli* strain BL21(DE3) (Novagen). Cultures were grown in

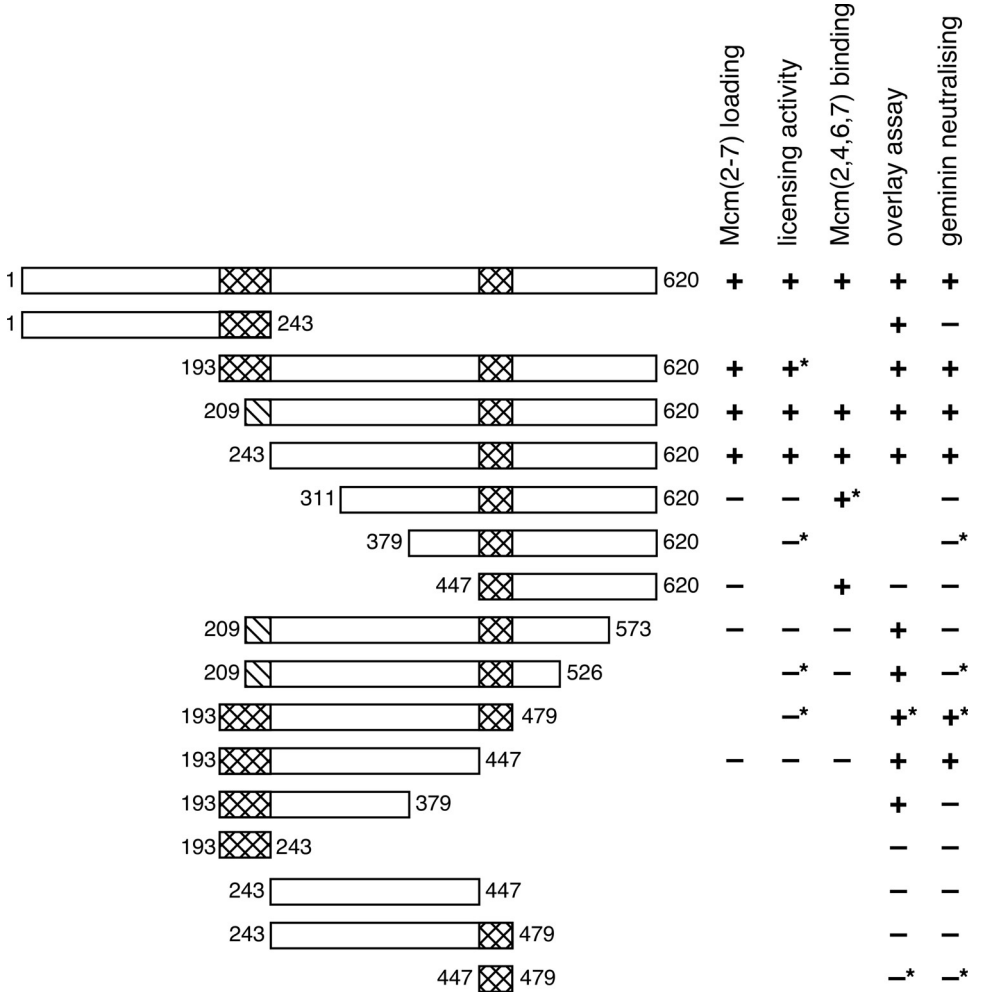

Figure 1. Summary of constructs used in this paper. The indicated regions of *Xenopus* Cdt1 were expressed in *E.coli* as GST fusions. The two regions predicted to form coiled-coils are indicated with cross hatching. Where deletions are predicted to significantly disrupt coiled-coil formation, the cross-hatching is replaced by diagonal shading. Results for the assays outlined in subsequent figures are summarized on the right. Data not explicitly shown in this paper are indicated by asterisk.

Luria–Bertani plus appropriate antibiotics to an OD_{600} of 0.6–0.7 before the addition of 1 mM isopropyl-β-D-thiogalactopyranoside and grown for a further 3 h. The cells were lysed using Bugbuster (Novagen) according to the manufacturer's instructions.

GST-Cdt1(193–243) and GST-Cdt1(447–479) were soluble and were bound to glutathione Sepharose 4B (Pharmacia) according to the manufacturer's instructions and eluted with 50 mM Tris–HCl, pH 8, plus 10 mM glutathione. All other GST fusion proteins were present in inclusion bodies and were solubilized using a detergent cancellation technique (24,25). The resulting protein preparation was then bound to glutathione Sepharose 4B as described above but with an additional second series of elution steps using 50 mM Tris–HCl, pH 8, 200 mM KCl, 8 M Urea, 10 mM glutathione and 0.03% Triton. Eluted protein was dialysed in Tube-O-Dialyser tubes (Geno-Tech) against 100× vol of THED 200 (0.03% Triton, 20 mM HEPES, pH 8, 20% ethylene glycol, 1 mM DTT and 200 mM KCl) containing progressively lower concentrations of urea, 4 M, overnight, then 2 h at 2 M, 1 M and two steps without urea. All constructs described in this paper are GST-tagged, except where explicitly stated.

His-tagged geminin^{DEL} (13) and the complex of His-Cdt1(193–447) and S-tagged mini-geminin (residues 87–168) were purified using Ni-NTA agarose beads (Qiagen) added directly to the protein suspension produced following Bugbuster treatment (supplemented with 10 mM imidazole). Beads were washed thoroughly with phosphate-buffered saline (PBS) containing 20 mM imidazole and 300 mM KCl. Proteins were eluted using the same buffer but containing 250 mM imidazole.

When expressed in the absence of geminin, recombinantly expressed His-Cdt1(1–620), His-Cdt1(193–447) and His-Cdt1(243–620) were present in inclusion bodies and were solubilized using IMAC5 (20 mM Tris, pH 8, 0.5 M NaCl, 5 mM imidazole and 8 M urea). Ni-NTA agarose beads were added to the solubilized inclusion bodies and a purification similar to that for soluble His-tagged proteins was followed, with 8 M urea present in all buffers. Elutions containing the highest concentration of protein were dialysed into THED 200 in the same manner as the insoluble GST fusion proteins.

To quantify protein concentration, samples were run on SDS–PAGE and stained with Coomassie; gels were scanned on a flatbed scanner and the images quantified using AIDA software version 3.27.001 (Fuji).

Chromatin isolation

For immunoblotting experiments, 20 µl aliquots of Cdt1-depleted extract were combined with dilutions of GST-Cdt1 constructs and supplemented with demembranated sperm nuclei at a final concentration of 10 ng DNA/µl extract (~3000 nuclei/µl). After a 30 min incubation, each reaction was diluted in 500 µl NIBA (50 mM KCl, 50 mM HEPES KOH, pH 7.6, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 2.5 mM Mg-ATP, and 1 µg/ml each of leupeptin, pepstatin and aprotinin) supplemented with 0.1% Triton X-100 and under-layered with 100 µl of the same buffer containing 15% sucrose. The chromatin was pelleted at 2100 *g* in a swinging bucket centrifuge for 5 min at 4°C. The diluted extract and the majority of the overlying cushion were then removed, and the chromatin was re-centrifuged at 13 000 *g* for 2 min in a fixed-angle rotor. The chromatin pellet was resuspended in loading buffer and subjected to immunoblotting by standard techniques using 4–12% Bis-Tris gradient SDS–PAGE (Invitrogen) and enhanced chemiluminescence (ECL) detection (SuperSignal® West Pico Chemiluminescent).

Two-step licensing assay

A two-step licensing assay specific for Cdt1 activity was used based on a previously described two-step assay (23,26). Sperm nuclei were first incubated in a small volume of Cdt1-depleted extract (which provides nucleoplasmin, ORC, Cdc6 and Mcm2-7 activities) supplemented with various Cdt1 constructs. The ability of the DNA to subsequently replicate in whole extract supplemented with active recombinant geminin reflects its degree of licensing. This is formally equivalent to the previously validated 6-DMAP assay (23,26) since we have recently shown that the only replication defect in 6-DMAP-treated extracts is the presence of active geminin (27). Aliquots containing 1 µl samples of GST-Cdt1 constructs were combined with 1.1 µl of Cdt1-depleted extract containing 24 ng DNA sperm nuclei. For the control reactions, the depleted extract was replaced by 1.1 µl buffer LFB2/50 (40 mM HEPES KOH, pH 8.0, 20 mM K_2HPO_4/KH_2PO_4, pH 8.0, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 10% (w/v) sucrose, and 1 µg/ml each of leupeptin, pepstatin and aprotinin, 0.1 mM phenylmethlysulfonyl fluoride, 2.5 mM ATP and 50 mM KCl) containing the same quantity of sperm. Reactions were incubated for 30 min at room temperature. An aliquot of 5.9 µl extract supplemented with 60 nM geminin^{DEL} and [α-^{32}P]dATP was added to the reactions and incubated for a further 90 min. Total DNA synthesis was measured by TCA precipitation.

Mcm(2,4,6,7) binding assay

The Mcm(2,4,6,7) complex was purified as described previously (28). A 20% slurry of glutathione Sepharose beads was prepared in Hybrid buffer (2.5 mM ATP, 0.02% Triton, 4 mg/ml BSA, 100 mM KCl, 20 mM HEPES, 10% sucrose, 2 mM DTT, 10% imidazole, and 1 µg/ml leupeptin, aprotinin and pepstatin). 50 µl aliquots were supplemented with 140 nM GST-tagged Cdt1 constructs and 5 nM purified Mcm(2,4,6,7). GST protein alone was used as a negative control. Reactions were incubated in 2 ml (square bottomed) Eppendorf tubes on a rotating mixer at 6 r.p.m. for 30 min. The tubes were spun down at 1000 *g* for 5 min, the supernatant was removed and two rinses of 100 µl Hybrid buffer were carried out, with spins between. An aliquot of 30 µl SDS loading buffer was applied directly to the beads, boiled for 5 min and 20 µl samples were loaded onto a gel and immunoblotted.

Overlay assay

Concentrations of constructs were standardized for the same band intensity on an SDS gel stained with Coomassie. Those concentrations were blotted to a nitrocellulose membrane. After blocking in PBS + 0.02% Tween + 4% milk, the membrane was then placed in a 50 ml Falcon tube containing 5 ml LFB2/50 containing 15 nM geminin^{DEL} and 0.4% BSA. The membrane was then blotted with rabbit anti-geminin antibody and treated subsequently in a manner identical to a standard

western blot. Cdt1(1–620) was used as a positive control, but at a 37-fold lower concentration than the other proteins. This dilution was used in order to normalize the signal intensity to that of the other constructs.

Gel filtration and glycerol gradients

4 ml linear 10–30% glycerol gradients were formed in 20 mM Tris–HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5 using a gradient mixer coupled to a peristaltic pump. Aliquots containing 200 µl samples of proteins of interest were applied to the top of the gradient and centrifuged at 42 000 *g* for 16 h in a SW60 rotor (Beckman). Aliquots containing 200 µl fractions were carefully collected from the top of the gradient using a pipette.

Gel filtration was performed essentially as described previously (16). Protein samples were applied to a 3.2 Superose 6 column equilibrated with TLFB1/200 (40 mM HEPES KOH, pH 8, 20 mM K_2HPO4/KH_2PO4, pH 8.0, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 1 µg/µl aprotinin, pepstatin and leupeptin, 10% sucrose, 0.03% Triton and 200 mM KCl) at a flow rate of 50 µl/min. Aliquots containing 30 fractions of 50 µl were collected.

Molecular weight standards used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13 kDa).

RESULTS

In order to investigate the structure and function of *Xenopus* Cdt1, a range of GST-tagged constructs were designed. The construct design was based on two regions predicted to form coiled-coils (probabilities of 0.92 and 0.31 using MacStripe v2.0) at residues 193–243 and 447–479, respectively. Out of a total of 21 constructs, 17 generated informative results and are described in this study (Figure 1).

Licensing activity of Cdt1 regions

The main activity that has been described for Cdt1 is as a component of the pre-RC that loads Mcm2-7 onto DNA to license it for subsequent replication ('RLF-B' activity) (4,5,7,26,29). We first determined the regions of Cdt1 that are essential for this licensing activity. Sperm nuclei were incubated in extract immunodepleted of endogenous Cdt1 supplemented with the different Cdt1 constructs; activity was then assessed by either determining whether Mcm2 and Mcm6 had been loaded onto chromatin (Figure 2A) or whether the chromatin could subsequently replicate when transferred to extract containing an excess of active geminin (Figure 2B). The results of both these assays were in agreement: the N-terminal 243 amino acids were dispensable for licensing activity, but residues between 243 and 311 were essential; residues close to the C-terminus were required for licensing activity, since deletion of the C-terminal 47 amino acids abolished activity. Residues 243–620 therefore represented the smallest construct possessing licensing activity.

Previous work has suggested that licensing involves the recruitment of Mcm2-7 to chromatin by direct interaction with Cdt1 (11,12). Yanagi *et al*. (11) showed that mouse Cdt1 possessed a binding site for Mcm6 in its C-terminus.

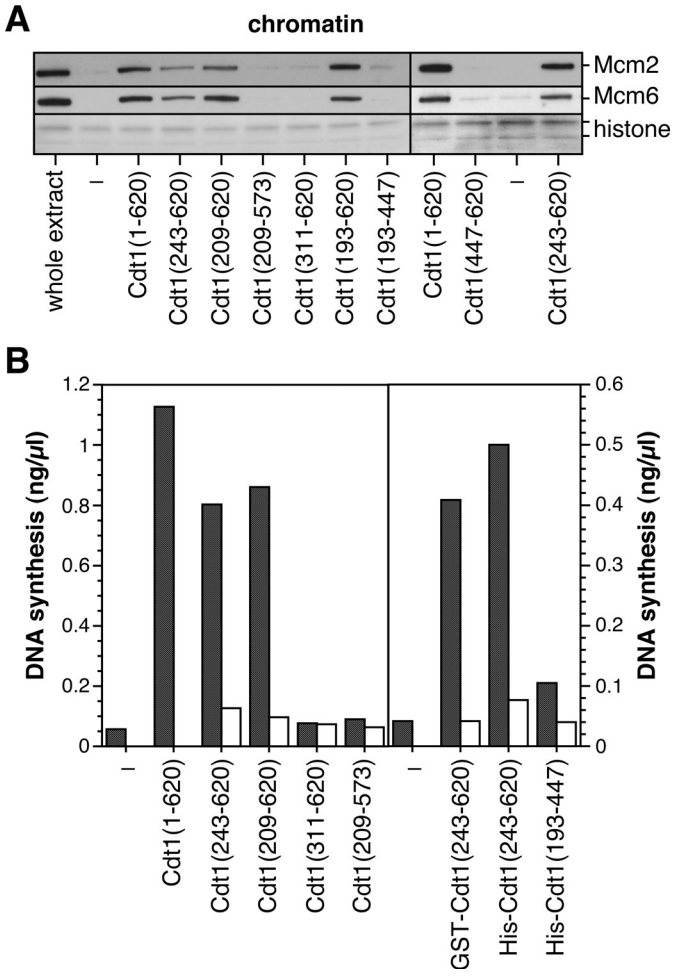

Figure 2. Licensing assay. (**A**) Interphase *Xenopus* egg extract or Cdt1-depleted extract was supplemented with *Xenopus* sperm nuclei (10 ng DNA/µl) and different GST-Cdt1 constructs at 5 nM. After incubation for 30 min, chromatin was isolated and immunoblotted for the presence of Mcm2 and Mcm6. (**B**) Cdt1-depleted extract was supplemented with *Xenopus* sperm nuclei (11.5 ng DNA/µl) and different GST-Cdt1 constructs at 42 nM (diagonal shading). After incubation for 30 min, aliquots were supplemented with 2.8 vol of *Xenopus* egg extract supplemented with [α-^{32}P]dATP and 60 nM geminin. After 90 min, total DNA synthesis was assessed by TCA precipitation. As controls for the sufficiency of geminin inhibition, assays were also performed, where the Cdt1-depleted extract was replaced with buffer (white boxes). The right-hand panel also shows the activity of two His-tagged Cdt1 constructs (243–620 and 193–447).

We therefore prepared a purified complex consisting of Mcm2, 4, 6 and 7 (28) and used this to perform pull-down experiments with different *Xenopus* Cdt1 constructs. The purified Mcm(2,4,6,7) complex is shown in Figure 3A. Figure 3B shows that like mouse Cdt1, full-length *Xenopus* Cdt1 can bind to Mcm(2,4,6,7). The binding activity resides in the extreme C-terminal 173 amino acids of *Xenopus* Cdt1, a region that is necessary, but not sufficient, for the licensing activity of Cdt1 (Figure 2). This is consistent with the idea that to function in licensing, Cdt1 needs bind Mcm2-7, but also needs other functions such as binding to ORC.

Geminin-binding activity of Cdt1 regions

The activity of Cdt1 is negatively regulated by binding geminin (7,14,15), so we next set out to identify the regions

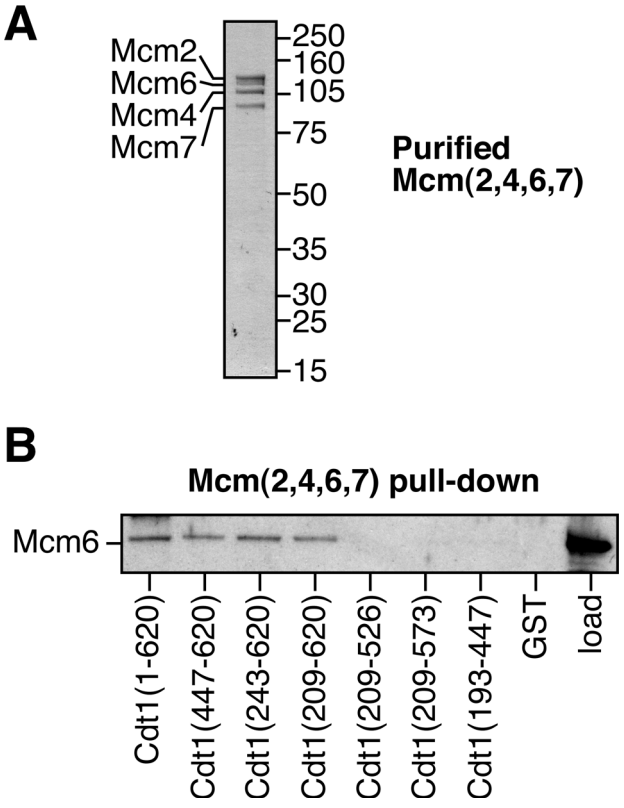

Figure 3. Mcm2-7 binding assay. (**A**) Coomassie-stained gel of purified Mcm2-7 used in the binding assay. The migration of molecular weight markers is shown to the right (size in kDa). (**B**) Glutathione–Sepharose beads were incubated for 30 min with 5 nM purified Mcm(2,4,6,7) and 140 nM GST-tagged Cdt1 constructs. After washing, bead-bound protein was eluted and immunoblotted for the presence of Mcm6.

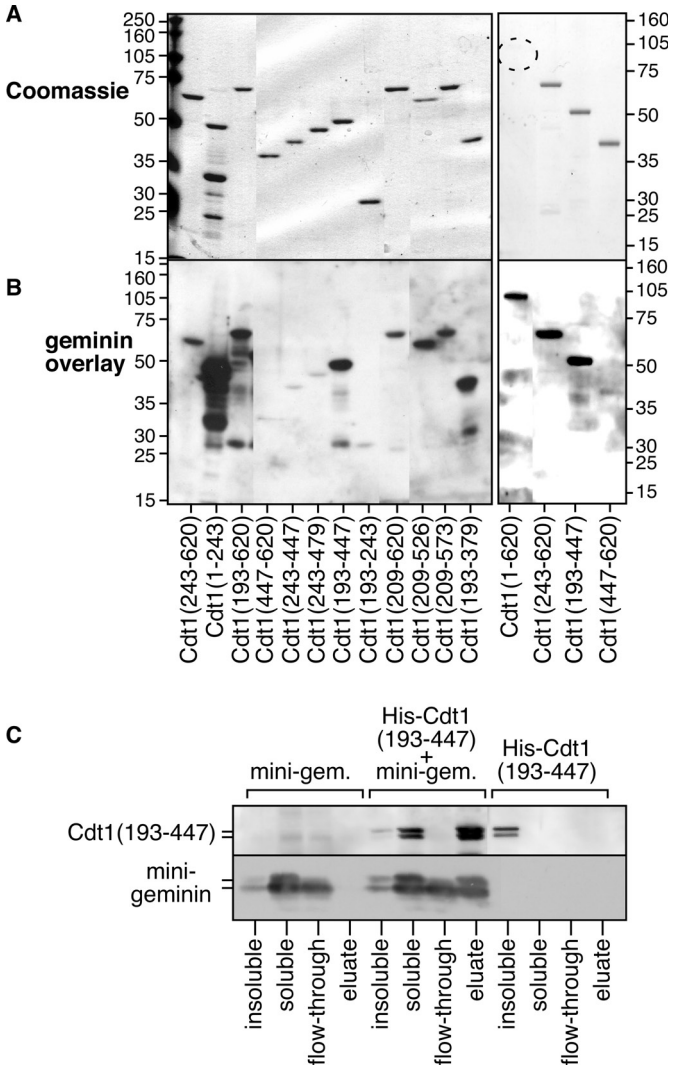

Figure 4. Overlay (far-western) assay. (**A** and **B**) Different GST-Cdt1 constructs were separated by SDS–PAGE. (A) Gels were stained with Coomassie. Molecular weight markers (sizes in kDa) are shown to the left. (B) Proteins were blotted onto nitrocellulose. After blocking, the nitrocellulose was incubated with recombinant geminin^{DEL}, followed by anti-geminin antibody and then anti-rabbit secondary antibody coupled to horseradish peroxidase. Bound geminin was revealed by ECL chemiluminescence. To give a comparable signal, Cdt1(1–620) was used at 37-fold lower concentration than the other fragments and was not visible by Coomassie staining; the position of the protein is indicated by a dashed oval. (**C**) His-tagged Cdt1(193–447) and an S-tagged version of geminin capable of binding Cdt1 (mini-geminin) were expressed together and separately in *E.coli*. After protein expression, cells were lysed and the proteins separated into soluble and insoluble components. Soluble protein was applied to nickel-NTA columns; the flow-through was collected, and then bound protein was eluted with imidazole. Fractions were separated by SDS–PAGE and immunoblotted for Cdt1 and geminin.

of *Xenopus* Cdt1 involved in geminin binding. Initial attempts to define the interacting regions by pull-down assay were inconclusive, probably due to the hydrophobicity of Cdt1 that tends to encourage non-specific interactions (7). To overcome some of these problems, we performed an overlay (far-western) assay. The Cdt1 constructs (Figure 4A) were blotted onto nitrocellulose and then incubated with recombinant geminin. The binding of geminin to the constructs was revealed using anti-geminin antibodies (Figure 4B). This assay showed that at least two different regions of Cdt1 were capable of interacting with geminin. The first region lies between the two predicted coiled-coil domains; binding additionally required part of either of the two predicted coiled-coil domains. The second region, somewhat surprisingly, comprises the N-terminal 243 amino acids. The first lane in the right-hand panel of Figure 4A and B shows that in order to give a comparable signal in the overlay assay, full-length Cdt1(1–620) had to be used at a 37-fold lower concentration than the shorter fragments containing one or other of the geminin-binding domains. This suggests that both domains cooperate to bind geminin. A recent paper has also implicated an additional N-terminal domain in geminin binding (30).

We next investigated in more detail the binding of geminin to Cdt1(193–447), which represents one of the shortest regions of Cdt1 capable of binding geminin in the overlay assay. For these experiments, we used a shortened version of geminin ('mini-geminin'), which is able to bind Cdt1 and inhibit licensing in *Xenopus* egg extracts (13). His-tagged Cdt1 (193–447) and mini-geminin were expressed together or separately in *E.coli*. After cell lysis, proteins were separated into soluble and insoluble fractions. Figure 4C shows that mini-geminin is found in the soluble fraction of *E.coli* lysates. When applied to a nickel column, it was found exclusively in the flow-through since it does not contain a His tag. In contrast,

when His-Cdt1(193–447) was expressed on its own, it partitioned almost exclusively into the insoluble fraction. Only when co-expressed with mini-geminin was it found in the soluble fraction. Furthermore, when co-expressed with His-Cdt1(193–447), mini-geminin bound to a nickel column, despite not possessing a His tag of its own. These results show that Cdt1(193–447) and mini-geminin can form a stable complex. Both proteins ran as doublets on SDS–PAGE; western blot analysis of the bands for mini-geminin showed that the lower band had lost the S-tag, while the upper band still contained the S-tag (data not shown). Mass spectrometry of the Cdt1(193–447) doublet revealed that the lower band had lost ~10 amino acids from its C-terminus (data not shown).

In order to identify which of the regions of Cdt1 capable of binding geminin in the overlay assay are physiologically important in binding geminin, we devised a competition assay. *Xenopus* egg extract was supplemented with sufficient recombinant geminin to completely abolish DNA replication; recombinant Cdt1 constructs were then added to the geminin-containing extract to compete with the endogenous Cdt1 for geminin binding, and the extracts were then assayed for their ability to support DNA replication. In principle, there are two ways that the Cdt1 constructs could induce DNA replication in this assay: they might provide licensing activity themselves, or they may be able to compete with endogenous Cdt1 for geminin binding, thereby liberating the endogenous Cdt1 and allowing it to induce DNA replication. Figure 5A shows that, consistent with this idea, all the constructs that possessed licensing activity (marked with an asterisk) were able to support efficient licensing in this assay. The N-terminal 243 amino acids, which lack licensing activity but bound strongly to geminin in the overlay assay, were not able to compete with the binding of endogenous full-length Cdt1. In contrast, residues 193–447, consisting of the predicted N-terminal coiled-coil plus the region between the predicted coiled-coils, induced efficient DNA synthesis although the construct itself possessed no licensing activity. This suggests that this region represents a domain that interacts strongly enough with geminin to displace full-length endogenous Cdt1 from its inhibitory interaction with geminin.

Taken together, the results shown so far suggest that the predicted N-terminal coiled-coil domain of *Xenopus* Cdt1 (residues 193–243) is required for tight binding to geminin, but is not required for licensing activity. Therefore, constructs lacking the predicted N-terminal coiled-coil domain but possessing licensing activity (i.e. residues 243–620) might be at least partially resistant to inhibition by geminin. To investigate this, *Xenopus* egg extract was supplemented with either full-length Cdt1 (residues 1–620), Cdt1(243–620) or the tight geminin binder Cdt1(193–447), plus increasing concentrations of geminin; the extracts were then assayed for their ability to support DNA synthesis. Figure 5B shows that although full-length Cdt1 or Cdt1(193–447) could rescue DNA synthesis in the presence of low concentrations of geminin, rescue was abolished at higher geminin concentrations. In contrast, Cdt1(243–620), which lacks the predicted N-terminal coiled-coil but possesses licensing activity, could still rescue replication even at concentrations of geminin 16-fold higher than were required to inhibit the other Cdt1 constructs capable of binding to geminin. This suggests that the licensing activity of Cdt1(243–620), which lacks the predicted N-terminal coiled-coil domain, is at least partially resistant to inhibition by geminin.

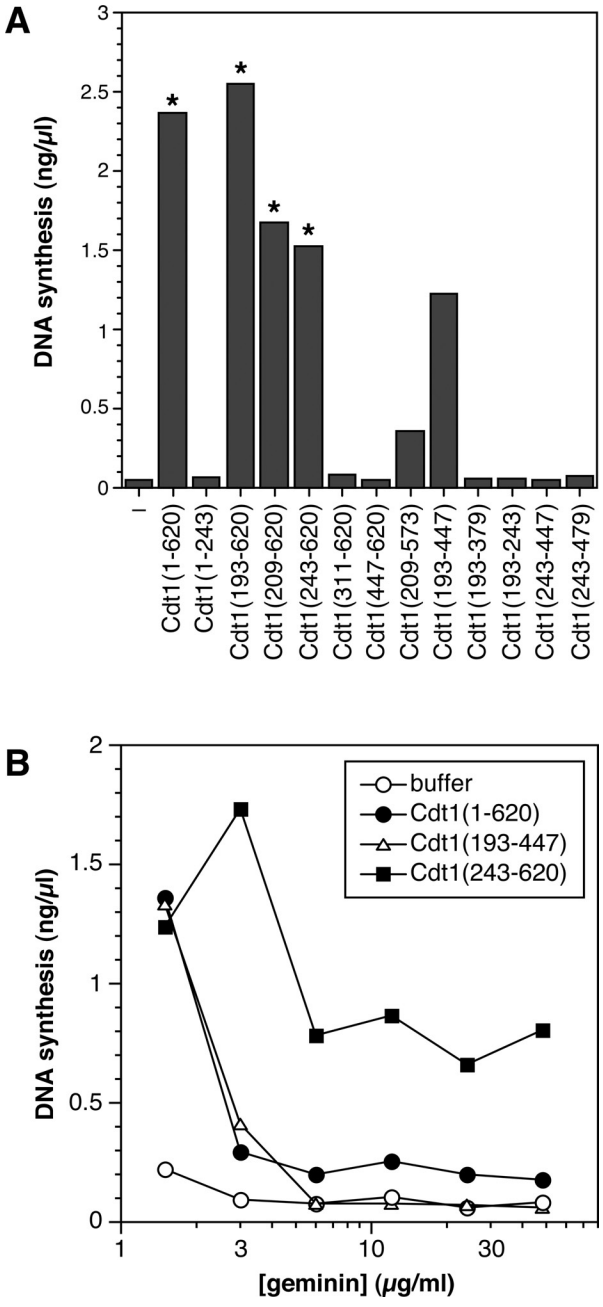

Figure 5. Geminin neutralization. (**A**) *Xenopus* egg extract was supplemented with sperm nuclei (3ng DNA/μl), 60 nM geminin^{DEL}, [α-^{32}P]dATP and 50 nM GST-Cdt1 constructs. After incubation for 90 min, total DNA synthesis was assessed by TCA precipitation. Asterisked columns show constructs shown to possess licensing activity. (**B**) *Xenopus* egg extract was supplemented with sperm nuclei (3 ng DNA/μl), [α-^{32}P]dATP, various concentrations of geminin^{DEL} and either 6.5 nM His-Cdt1(1–620) (black circles), 7.1 nM His-Cdt1(243–620) (black squares), 7.7 nM His-Cdt1(193–447) (white triangles) or buffer alone (white circles). After incubation for 90 min, total DNA synthesis was assessed by TCA precipitation.

Stoichiometry of the geminin–Cdt1 complex

We next set out to determine the stoichiometry of geminin and Cdt1 when complexed together. Gel filtration of recombinant

geminin showed it eluting with an apparent molecular weight of ~300 kDa (Stokes' radius ~54 Å), similar to the behaviour of endogenous geminin in *Xenopus* extract (16,27) (data not shown). Glycerol gradient sedimentation of geminin^{DEL} showed it migrating with an apparent molecular weight of ~25 kDa (sedimentation value ~2.5×10^{-13} S). Combining these values in the equation of Siegel and Monty (31) gives a value of 56 600 kDa for the native molecular weight of geminin, suggesting that it exists as a highly elongated dimer, which is consistent with a number of recent reports (22,30,32,33). We next co-expressed various constructs of His-tagged Cdt1 and geminin in *E.coli* in order to produce soluble complexes containing both proteins. Almost all of the complexes we produced were insoluble or unstable. We did, however, manage to produce reasonable quantities of a complex between the tight geminin binder Cdt1(193–447) and mini-geminin. On gel filtration, the complex of both proteins eluted at an apparent molecular weight of ~100 kDa (Stokes' radius ~40 Å; Figure 6A). A small amount of protein with a larger size was also observed. When the peak fraction was immunoprecipitated with anti-geminin antibodies, Cdt1(193–447) was efficiently co-precipitated, confirming that this represents a complex between mini-geminin and Cdt1(193–447) (Figure 6B). On glycerol gradient sedimentation, the complex of both proteins migrated at an apparent molecular weight of ~50 kDa (sedimentation value ~3.6×10^{-13} S; Figure 6C), again with a small amount of protein at a larger size. The Siegel and Monty equation suggests that the major peak of protein represents a slightly elongated complex with a molecular weight of ~60 kDa. Monomeric Cdt1(193–447) has a predicted molecular weight of 30 kDa, while monomeric mini-geminin has a predicted molecular weight of 11.5 kDa. A complex consisting of one molecule of Cdt1(193–447) and two molecules of mini-geminin would therefore have a combined molecular weight of 53 kDa, close to the derived value. Control experiments showed that on its own, the Cdt1(193–447) construct was a monomer (Stokes' radius ~35 Å, sedimentation value ~2×10^{-13} S; data not shown). To further support our interpretation of the make-up of the complex, we examined the composition of the complex on SDS–PAGE gels stained with Coomassie (Figure 6D). The relative intensities of the bands were measured, giving a value of 0.72 for the ratio of mini-geminin: Cdt1(193–447). Since the ratio of their molecular weights is 0.38, the molar ratio of the proteins in the complex is ~1.9 mini-geminin: 1 Cdt1(193–447). This is consistent with the complex consisting of two molecules of mini-geminin and one molecule of Cdt1(193–447).

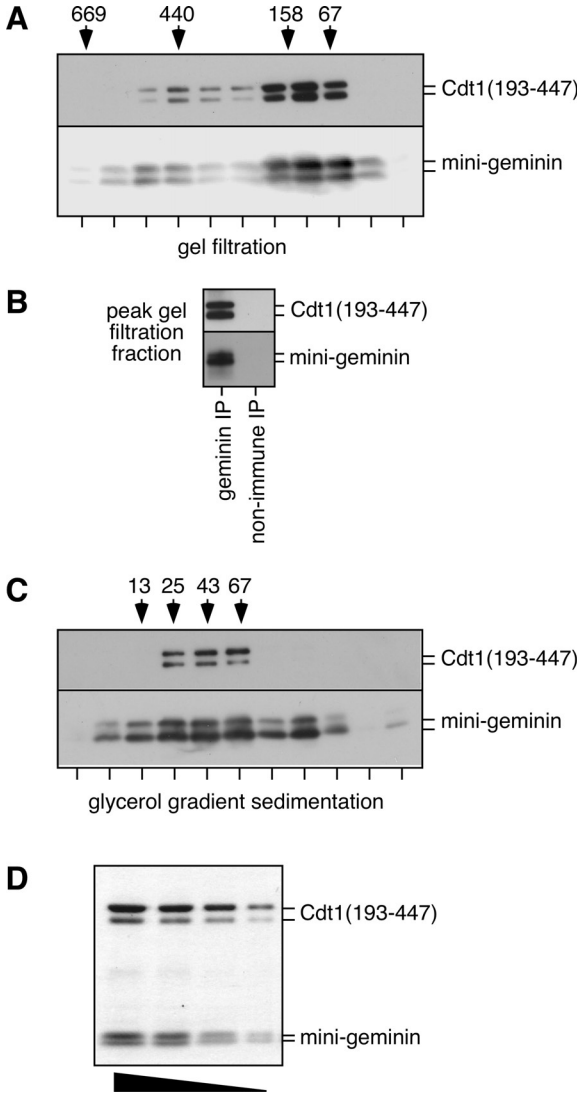

Figure 6. Characterization of complexes between Cdt1(193–447) and mini-geminin. Recombinant complexes between His-Cdt1(193–447) and His-tagged mini-geminin were prepared. (**A** and **B**) Complexes were subject to gel filtration. (A) Fractions were subjected to SDS–PAGE and immunoblotted for Cdt1 and geminin. The migration of molecular weight markers (sizes in kDa) is also shown. (B) The peak fraction of geminin and Cdt1 from the gel filtration column was immunoprecipitated with either anti-geminin antibodies or non-immune antibodies; precipitates were then immunoblotted for Cdt1 and geminin. (**C**) Complexes were subject to glycerol gradient sedimentation. Fractions were subjected to SDS–PAGE and immunoblotted for Cdt1 and geminin. The migration of molecular weight markers (sizes in kDa) is also shown. (**D**) Different quantities of the complex were subjected to SDS–PAGE and stained with Coomassie.

DISCUSSION

In the work presented here, we have mapped a number of functional domains on *Xenopus* Cdt1. Our conclusions are summarized in Figure 7A. The main function of Cdt1 is to load Mcm2-7 onto chromatin to license the DNA for replication in the subsequent S phase. We show here that the C-terminal 377 residues (amino acids 243–620) are required for licensing activity. Licensing probably involves the direct interaction between Cdt1 and Mcm2-7 (11,12). We show here that the C-terminal 173 residues (amino acids 447–620) are required for Cdt1 to interact with an Mcm(2,4,6,7) complex. This is consistent with data from mouse Cdt1, showing that the C-terminal 222 residues (corresponding to *Xenopus* Cdt1 residues 398–620) are necessary for interaction with Mcm6 (11). It has been proposed that geminin inhibits Cdt1 function by hindering access of Mcm2-7 to this binding site (22). Although the Mcm binding domain is necessary for licensing activity, we show here that other regions of Cdt1 further to the N-terminus are also required. This is plausible, since in addition to binding Mcm2-7, Cdt1 presumably also needs to bring them to ORC and Cdc6 on the DNA. It has previously been shown that Cdt1 is directly recruited to DNA by ORC, and we

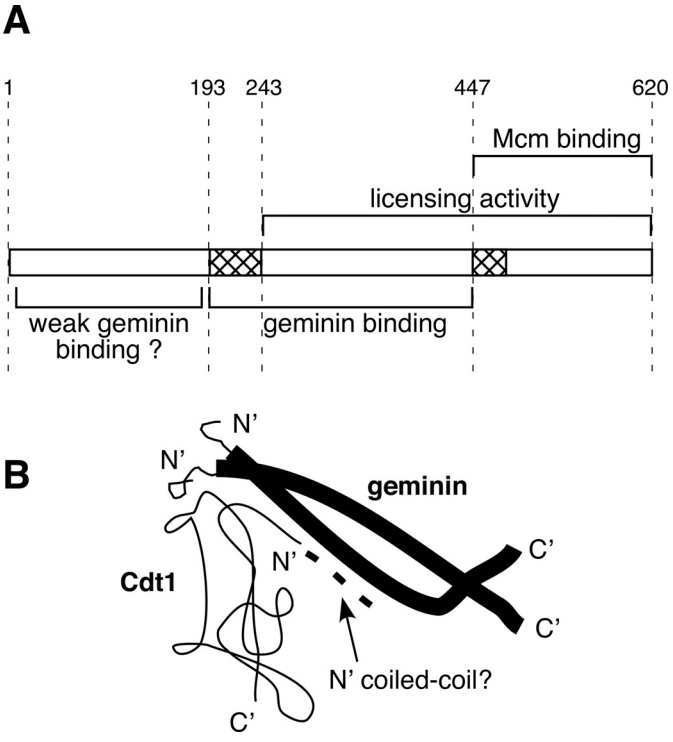

Figure 7. Model of Cdt1 domains. (**A**) Schematic diagram showing the minimum domains necessary for Mcm binding, licensing activity and geminin binding. Predicted coiled-coil domains are shown with hatching. (**B**) Diagram of the structure of the Cdt1-geminin interface redrawn from reference (22). The fragment of Cdt1 corresponds to *Xenopus* Cdt1 residues 232–426. The proposed alignments of Cdt1 residues 193–231, which are predicted to form a coiled-coil, are shown as a dashed line. The coiled-coil section of geminin is shown with heavy lines.

propose that amino acids 243–447 of Cdt1 are required for this recruitment.

Cdt1 is mainly controlled by binding to geminin, which inhibits Cdt1's licensing activity (7,14). In metazoans, down-regulation of Cdt1 by geminin is a major mechanism preventing re-replication of DNA in a single cell cycle (7,16,17,20,34). We show here, using an overlay assay, that at least two regions of *Xenopus* Cdt1 can interact with geminin: a region between the predicted coiled-coil domains, and a region at the N-terminus of the protein. Full-length Cdt1 bound geminin much more avidly than either domain alone. Work on human Cdt1 also provides evidence for two different geminin-binding domains in these regions (11,30,35,36). However, we show here that only the central region (amino acids 193–447), which includes the predicted N-terminal coiled-coil domain, is capable of displacing geminin from endogenous Cdt1 in a functional licensing assay. The predicted N-terminal coiled-coil domain, while essential for a high affinity interaction with geminin, is not required for licensing activity. Consistent with these ideas, Cdt1(243–620), which is the minimal construct possessing licensing activity, was at least partially resistant to inhibition by geminin. This construct therefore has potential use in exploring the *in vivo* consequence of uncontrolled Cdt1 activity.

While we were preparing this work for publication, several papers describing the structure of Cdt1 and geminin were published (22,30,33,37). One of these papers describes a crystal structure of a fragment of mouse geminin in complex with a central region of Cdt1 (22). The region of mouse Cdt1 co-crystallized with geminin contained amino acids 172–368, which corresponds to *Xenopus* Cdt1 residues 232–426. The structure is likened to that of an axe, where geminin forms the shaft and the Cdt1 fragment forms the blade (Figure 7B). Two other papers present a crystal structure of geminin on its own, showing a long coiled-coil dimer (30,33). Consistent with the crystal structures, we provide evidence from glycerol gradient sedimentation and gel filtration that geminin forms a highly elongated homodimer, and that each dimer of geminin can interact with one molecule of Cdt1. Another recent paper provides evidence that two geminin dimers can complex together to form a tetramer (37). Although we find no evidence of this tetrameric structure, it is possible that the dimers interact only weakly, and so the interaction is substantially disrupted during gel filtration and glycerol gradient sedimentation.

The geminin–Cdt1 co-crystal shows a loop and a short helix at the end of the geminin fragment, as well as the N-terminal portion of the coiled-coil, interacting with the Cdt1 fragment (22). From the results we present here, the *Xenopus* region corresponding to the Cdt1 fragment in the co-crystal would not be expected to bind to geminin tightly enough to displace endogenous Cdt1 in the geminin neutralizing assay. For this physiological interaction to take place, the predicted N-terminal coiled-coil domain of Cdt1 is additionally required. This predicted coiled-coil domain of Cdt1 is perfectly positioned to form a triple coiled-coil interaction with the geminin 'shaft', which would be expected to significantly strengthen the interaction between the two proteins (Figure 7B). In support of this idea, residues in geminin towards the middle of the shaft enhance the binding with Cdt1 and are essential for inhibiting DNA replication (13,22,30,32,33). Like *Xenopus* Cdt1, both human and mouse Cdt1 have a region of predicted coiled-coil just N-terminal to the 'blade' fragment of Cdt1 (amino acids 113–152 in human Cdt1 and amino acids 126–163 in mouse Cdt1), consistent with the idea that this is a conserved feature of metazoan Cdt1.

Previous work has shown that when overexpressed, geminin is capable of selectively killing cancer cells (38,39). It is therefore of considerable interest to understand how geminin inhibits Cdt1. In providing further details of the geminin–Cdt1 interaction, the current work will help the design of molecular mimics that display geminin's capacity for cancer-specific cell killing.

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