The Replicative Regulator Protein Geminin on Chromatin in the HeLa Cell Cycle*

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Geminin is believed to have a major function in the regulation of genome replication and cell proliferation. Published evidence shows that geminin specifically interacts with Cdt1 to block its function in the assembly of prereplication complexes. However, in proliferating HeLa cells geminin and Cdt1 are co-expressed during a relatively short time at the G1-to-S phase transition. Under these conditions, nearly all Cdt1 and a major part of geminin are bound to chromatin and reside at the same or closely adjacent sites as shown here by chromatin immunoprecipitation. Cdt1 is rapidly degraded early in S phase, but geminin remains bound to the chromatin sites. One function that chromatin-bound geminin could perform is to prevent access to Cdt1 that may escape S phase-depending degradation or is synthesized in excess. Indeed, Cdt1 continues to be synthesized in HeLa cells in S phase but never accumulates because of the efficient degradation. Therefore, geminin can be eliminated by RNA interference without detectable effects on cell cycle parameters.

Geminin, a 25-kDa protein, was discovered by screening a cDNA library for novel proteins that were degraded by mitotic but not interphase Xenopus laevis egg extracts (1). Homologs of geminin contain a destruction box in the amino-terminal region that is targeted for ubiquitin-dependent proteolysis by the anaphase-promoting complex (APC) ubiquitin ligase activated in late mitosis. Homologs of the Xenopus geminin have been detected in all multicellular eukaryotes examined but have not been detected in yeast. The native protein is a homodimer with a central interacting coiled-coil domain. This domain is involved in interactions with other proteins (2).

A remarkable feature of geminin is its ability to prevent the licensing of Xenopus chromatin for DNA replication in vitro (1). This is achieved by a specific binding and inhibition of the protein Cdt1 that together with protein Cdc6 loads the Mcm protein complex to chromatin sites bearing the origin recognition complex (ORC)3 (3, 4). It has been proposed that the major function of geminin in vivo is to inhibit the relicensing of replication origins that have already been activated in the same S phase (5). This model would explain why geminin accumulates during S phase and why it is degraded at the end of mitosis when Cdt1 must be released to function as an Mcm loader for the formation of prereplication complexes in the next cell cycle.

However, ongoing research with the Xenopus system suggests that the situation may be not as straightforward as this model implies. First, unlike recombinant geminin as investigated originally by McGarry and Kirschner (1), endogenous geminin is only partially degraded during mitosis in egg extracts but appears to be inactivated by other means, probably also involving ubiquitination but without consecutive proteolysis (6, 7). Second, it seems that geminin must first bind to chromatin before it can exert its Cdt1-inhibiting activity in Xenopus egg extracts (8). Third, in vivo the level of geminin remains stable and does not fluctuate during the synchronized and rapid first embryonic cell cycles in Xenopus (8), and the expression of geminin can be suppressed without effects on the early cell cycles until the midblastula transition, when differentiation begins and cell cycles become longer and asynchronous (9).

In addition, geminin has been independently discovered as a protein that affects the differentiation of neural tissues in gastrulating Xenopus embryos (10) raising the possibility that geminin may be involved in the coordination of cell proliferation and differentiation. This notion is supported by more recent evidence. Del Bene et al. (11) detected an interaction between geminin and Six3, a member of the homeobox-containing Six family of proteins involved in vertebrate eye development. Loss of geminin promotes excess retinal precursor cell proliferation, whereas overexpression of geminin induces forebrain and eye defects in medaka fish. The basis for this is probably that geminin can interact either with Six3 to prevent differentiation or with Cdt1 to block replication. Likewise, Luo et al. (12) have identified a strong interaction between geminin and transcription factors of the Hox and polycomb families, which are involved in embryonic patterning and tissue differentiation. Again, it is proposed that a binding of geminin to Hox releases Cdt1 and thereby allows DNA replication and cell proliferation. Whatever the precise molecular mechanisms may be, it is interesting to note that geminin may have regulatory functions beyond prevention of relicensing as proposed originally.

Work with proliferating human HeLa cells in culture has shown that Cdt1 is expressed during G1 phase and then degraded soon after entry into S phase (13). This appears to be promoted by a cyclin A-dependent kinase, which phosphorylates Cdt1 and targets it to the F-box protein Skp2, a component of the Skp-Cullin-F-box protein ubiquitin ligase complex, SCF (14–17). In contrast, geminin is expressed at the entry into S phase (18, 19) and accumulates until its sudden destruction in late mitosis (19, 20). Thus, it is only for relatively short periods around the G1-to-S phase transition and at the end of mitosis that Cdt1 and geminin coexist in proliferating human cells.

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§ The abbreviations used are: ORC, origin recognition complex; ChIP, chromatin immunoprecipitation; RNAi, RNA interference; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; siRNA, small interfering RNA; 2×T, double-thymidine (procedure).
Interestingly, however, overexpression of a non-degradable geminin variant causes primary human cells to arrest before entry into S phase, whereas cultured cancer cell lines respond to overexpression with or without cell cycle arrest depending on whether they are able to activate a replication checkpoint or not. In either case, overexpression reduces the tumorigenicity of certain cultured cancer cells (20). Similarly, replacing the wild type geminin allele with a mutant allele expressing a non-degradable version of geminin causes a reduction in the tumor-promoting potential of HCT116 cancer cells, and this is explained by the fact that in this case the cell cycle is somewhat slower than normal but not altogether abolished (21). Although geminin acts to slow down cellular proliferation in vitro, it is highly expressed in numerous malignancies (22, 23) in contrast to other negative cell cycle regulators like cyclin-dependent kinase inhibitors, which are clearly down-regulated in many human tumors.

Thus, many questions remain before the roles of geminin in metazoan cell biology can be clearly defined. We have noted before that geminin is in part bound to chromatin in HeLa cells (24), and we investigate now whether geminin needs Cdt1, a known DNA-binding protein (25), to associate with chromatin or whether geminin also binds in the absence of Cdt1. We describe that a major fraction of geminin remains on chromatin in S phase after Cdt1 has been degraded, and using chromatin immunoprecipitation (ChIP), we have found that geminin is bound to sites adjacent to a mapped replication origin. We discuss the possibility that bound geminin may function to block the access of initiator proteins to replication origins. However, this function appears to be dispensable in rapidly proliferating HeLa cells because an elimination of geminin (by RNAi) has no apparent effect on cell division.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Fractionation**—HeLa cells were cultivated and synchronized by a double-thymidine block as described by Kreutz et al. (26).

For FACS analysis, HeLa cells were washed three times and then suspended in phosphate-buffered saline (PBS) supplemented with 5 mM EDTA. For staining of the DNA, 4 x 10^4 cells were permeabilized with 1% Triton X-100 and stained with a total of 18 μg of propidium iodide (Sigma). Fluorescence was measured with the flow cytometer FACS-Calibur (BD Biosciences), and results were visualized and evaluated with the software Cell Quest (BD Biosciences).

For cell fractionation cells were washed three times with hypotonic buffer (20 mM HEPES, pH 7.5, 20 mM NaCl, 5 mM MgCl₂, 1 mM ATP), suspended in the same buffer, and homogenized with a Dounce homogenizer five times before incubation on ice for 15 min. Cytosolic supernatant was separated from the nuclear pellet by centrifugation for 5 min at 600 x g. Soluble nuclear proteins were released with hypotonic buffer supplemented with 0.5% Nonidet P-40. Bound proteins were eluted from the chromatin pellet with increasing concentrations of salt (buffer containing 20 mM HEPES, pH 7.5, 0.5 mM MgCl₂, 1 mM ATP plus 100, 250, or 450 mM NaCl). All extraction buffers were supplemented with an EDTA-free protease inhibitor mixture (Roche Applied Science) in the concentration suggested by the manufacturer.

**In Vivo Cross-linking**—HeLa cells were washed with PBS to remove serum-containing medium and treated with 1% formaldehyde diluted in medium without serum at 37 °C. Cells were washed three times in ice-cold PBS to stop the reaction, scraped off, and washed again twice in cold PBS. Cells pelleted were then resuspended in hypotonic RSB buffer (10 mM Tris, pH 8, 3 mM MgCl₂) plus 10 mM sodium bisulfite as protease inhibitor, incubated on ice for 10 min, and disrupted mechanically by Dounce homogenization. Then nuclear pellets were washed twice with RSB buffer and once with high salt NSB buffer (1 M NaCl, 10 mM Tris, pH 8, 0.1% Nonidet P-40, 1 mM EDTA) plus 10 mM sodium bisulfite, and loaded onto a three-step gradient with 1.75, 1.5, and 1.3 mg/ml CaCl₂ in 20 mM Tris, pH 8, 0.5% sarcosyl, and 1 mM EDTA. After centrifugation nucleoprotein complexes were isolated.
and dialyzed for 1 h against TE (10 mM Tris, pH 7.4, 1 mM EDTA) plus 1 mM NaCl and 10 mM sodium bisulfite and for 4 h against TE buffer. After sonication nucleoprotein was digested with micrococcal nuclease (10 units/mg of nucleoprotein) in TE plus 3 mM CaCl₂ and 10 mM sodium bisulfite for 15 min at 37 °C.

Preparation and Use of Antibodies—Monospecific antibodies against human proteins geminin, Orc2, and Mcm7 were prepared previously (24, 26, 27). To prepare human Cdt1 as an antigen, we cloned a cDNA sequence (GenBank™/EBI accession number BE018212) into the multiple cloning site of phlbacHis2A (Invitrogen). Recombinant Histagged Cdt1 was expressed in Hi5 insect cells with recombinant baculoviruses, purified with nickel-nitritotriacetic acid (Qiagen), and used as an antigen to raise antibodies in rabbit. Monospecific antibodies were purified from serum by affinity chromatography with Sulfo-Link (Pierce) coupled with recombinant His-tagged Cdt1.

For immunoblotting proteins were separated by polyacrylamide gel electrophoresis (28) and transferred onto a Protran nitrocellulose transfer membrane (Schleicher & Schuell). The membrane was blocked with Roti® blocking reagent (Roth) for 1 h, stained with monospecific antibodies for 1 h, and visualized by goat anti-rabbit Ig (Jackson Immunoresearch Laboratories) with the enhanced chemiluminescence system (ECL) as suggested by the manufacturer (Amersham Biosciences). Immunoprecipitations were performed with 2 µg of monospecific antibodies/protein extracts from 4–6 × 10⁶ cells essentially as described previously (26).

For chromatin immunoprecipitations (ChIP assay) 10 µg of antibodies were incubated with 1 µg of micrococcal nuclease-digested nucleoprotein overnight at 4 °C in NET buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). Protein A-Sepharose beads were added for an additional 2 h. For Western blot analyses the immunocomplexes were washed five times with radioimmunoprecipitation assay buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS), once with lithium buffer (10 mM Tris, pH 8, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), and three times with TE buffer. For PCR analyses immunocomplexes were washed eight times with radioimmunoprecipitation assay buffer, three times with lithium buffer, and five times with TE buffer. The pellets in the buffers were supplemented with 10 mM sodium bisulfite and protease inhibitor. Immunocomplexes were eluted with 2% SDS (Western blot analysis) or 1% SDS in TE buffer (PCR analysis) at 37 °C for 30 min. Samples were incubated at 60 °C for 3 h to reverse the cross-links. Proteins were precipitated and subjected to SDS-PAGE for Western blot analysis, and DNA was extracted by standard phenol chloroform extraction, ethanol-precipitated, and dissolved in TE buffer for PCR analysis.

Quantitative Real Time PCR Analysis—Real time PCR was performed with the LightCycler instrument (Roche Diagnostics) using a ready-to-use "hot start" reaction mix (FastStart DNA Master SYBR Green I; Roche Diagnostics). Reactions were set up in a 10-µl volume with 0.5 mM primer oligonucleotides. PCR reactions were performed for 45 cycles with standard settings as recommended by the manufacturer. Sequences and conditions for each primer pair are exactly as described previously (29). Standard DNA samples were diluted from 30, 3, and 0.3 ng up to 30 pg. PCR results were evaluated against Cdt1, geminin, Mcm7, and Orc2 as shown. B. Immunoprecipitation from G1-to-S phase cells. Extracts containing chromatin-bound proteins from G1-to-S phase cells (16 h after release) were treated with antibodies against geminin or Cdt1. Input (I), supernantant (S), and precipitate (P) were analyzed for the presence of geminin and Cdt1 by Western blotting. One-third of the protein extract that was used for the immunoprecipitation was loaded for the input blot, and one-third was loaded for the supernatant blot.

RESULTS

Geminin and Cdt1 on Chromatin in Proliferating HeLa Cells—We have prepared monospecific antibodies against recombinant Cdt1 that recognize a single 63-kDa polypeptide band, corresponding to cellular Cdt1 in Western blots with unfractionated HeLa cell extracts (Fig. 1A). With these antibodies and the previously described geminin-specific antibodies (24) as tools, we investigated the distribution of Cdt1 and geminin in asynchronously proliferating HeLa cells. For this purpose HeLa cells were disrupted in a hypotonic buffer to prepare a cytosolic supernatant and a nuclear pellet. The pellet was suspended in the presence of the nonionic detergent Nonidet P-40 to disrupt the nuclear envelope. The supernatant fraction of nucleosolic proteins was separated from chromatin in the pellet. Finally, chromatin-associated proteins were mobilized from the pellet with increasing salt concentrations.

Nearly all Cdt1 was found in the chromatin fractions, whereas geminin distributed between the fractions of chromatin-bound proteins and soluble (cytosolic and nucleosolic) proteins (Fig. 1B). To determine whether Cdt1 and geminin interacted with each other, we performed coimmunoprecipitation experiments with the proteins eluted at 0.25–0.45 mM NaCl from chromatin. As shown in Fig. 1C, geminin-specific antibodies precipitated not only geminin, as expected, but a part of Cdt1 as well, just as Cdt1-specific antibodies partially coprecipitated geminin. This indicates that chromatin-bound Cdt1 interacted in part with geminin. However, other parts of either geminin or Cdt1 did not coimmunoprecipitate and were therefore not associated with each other. One explanation is that the complex was artifically disrupted during the experimental procedure, but it is also possible that geminin and Cdt1 bind in part independently of each other to chromatin. Furthermore a pro-
liferating cell population consists of cells in different cell cycle stages that are not all expected to co-express geminin and Cdt1. Thus it was of further interest to investigate synchronized cells with respect to the chromatin association as well as interaction of these proteins.

**Cdt1 and Geminin in Synchronized Cells**—To investigate how the chromatin binding of geminin and Cdt1 changes during the cell cycle, we arrested HeLa cells at the G1-to-S phase transition by the double-thymidine (2T) procedure (31). This involves the competitive inhibition of deoxynucleotide synthesis and therefore causes a cell cycle block that presumably occurs independently of and after the activation of cyclin E- and cyclin A-dependent protein kinases. Because cyclin A-dependent protein kinase targets Cdt1 for degradation, Cdt1 was not detected in 2T-arrested cells (13). A small amount of soluble Cdt1 was detected during mitosis at 8–10 h after release. This soluble Cdt1 had a retarded electrophoretic mobility that is most likely due to a phosphorylation by cyclin-dependent kinases. Because cyclin A-dependent protein kinase targets Cdt1 for degradation, Cdt1 was not detected in 2T-arrested cells (13). A small amount of soluble Cdt1 was detected during mitosis at 8–10 h after release. This soluble Cdt1 had a retarded electrophoretic mobility that is most likely due to a phosphorylation by cyclin-dependent kinases (see Fig. 2A, bottom, nucleosomal) (15, 17). Cdt1 continued to be expressed during G2, and most of it was bound to chromatin (Fig. 2A, top, elution with 450 mM NaCl).

In contrast to Cdt1, geminin was expressed in 2T-arrested cells (13), and a significant fraction was bound to chromatin even in the absence of Cdt1 (Fig. 2A, top). In parallel with soluble geminin (Fig. 2A, bottom), chromatin-bound geminin was degraded in mitosis and re-expressed at the beginning of the next S phase (Fig. 2A). As controls, we determined the presence of the Orc2 protein that is known to stabilize Cdt1 during the entire cell cycle (26) and of the Mcm7 protein that together with other Mcm proteins is known to dissociate during S phase and to bind to chromatin again in the next G1 phase (Fig. 2A) (32).

HeLa cells released from the 2T block partially lose synchrony during the next cell cycle (26). Nevertheless, a major fraction of cells at 14 and 16 h after the release appeared to co-express geminin and Cdt1 (see below). We have therefore prepared chromatin-bound proteins from the 16 h-cells and performed coinmunoprecipitation experiments to assess whether the two proteins occur together in complexes. The results indicate that parts, but not all, of Cdt1 and geminin were associated with each other (Fig. 2B) supporting the results obtained with unsynchronized cells (Fig. 1C).

**Cross-linking to DNA in Vivo**—We showed above that a significant part of cellular geminin was associated with isolated chromatin, even after the degradation of Cdt1, a characterized DNA-binding protein and an established binding partner of geminin. As isolated geminin has no DNA binding activity in vitro (25), it was necessary to exclude the possibility that geminin artifactually attached to chromatin after preparation. To show directly that geminin is a genuine constituent of chromatin in vivo, we used the method of in vivo formaldehyde cross-linking (33) and treated asynchronously proliferating HeLa cells with formaldehyde for various times. The chromatin was washed with high salt to remove all soluble proteins and isolated by CsCl density centrifugation. As described before (34) a cross-linking time of 1–2 min suffices to covalently link histones to DNA, and longer cross-linking times (4–8 min) are required to link most non-histone chromatin proteins (Fig. 3A, top). As shown by Western blotting, Cdt1 appeared in cross-linked chromatin already after 2–4 min in formaldehyde, soon followed by the cross-linking of geminin (Fig. 3A, Cdt1 and Geminin). Thus, geminin, just like Cdt1, appears to be an in vivo component of chromatin.

The question arises as to whether both proteins can reside at the same sites in chromatin. To investigate this, we trimmed the DNA in cross-linked chromatin by micrococcal nuclease digestion to fragments with average DNA lengths of <0.5 kb (Fig. 3B). These fragments were incubated with Cdt1-specific and, in parallel, with geminin-specific antibodies to immuno-
precipitate chromatin bearing covalently linked Cdt1 and/or geminin.

The Cdt1 antibodies only partially precipitated the cross-linked chromatin bearing Cdt1 (compare input (I) and supernatant (S) in Fig. 3C, center (α-Cdt1)). The fraction of immunoprecipitated chromatin did not increase with higher amounts of antibodies suggesting that not all of Cdt1 was accessible to antibodies; perhaps it was part of a larger complex as published recently (6, 8). The precipitated Cdt1-bearing chromatin fragments also carried geminin (Fig. 3C, center) indicating that the two proteins were located at identical or closely adjacent chromatin sites.

As shown in Fig. 3C (left) cross-linked geminin was accessible for geminin-specific antibodies and could be efficiently immunoprecipitated. However, Cdt1 was barely detectable in these precipitates. The reason is probably that much more geminin than Cdt1 was present on chromatin in these asynchronously growing cells with the consequence that many fragments carry geminin, but not Cdt1, which is therefore present in amounts too low to be detected in the precipitates obtained with geminin-specific antibodies.

To further address this point, we performed cross-linking experiments with synchronized cells that were treated with formaldehyde at 16 h after release from the 2×T block when Cdt1 and geminin are expected to be co-expressed (see Fig. 2A). Geminin-specific antibodies precipitated chromatin fragments with geminin, which also contained small but significant amounts of Cdt1 (Fig. 4A, left). Likewise, immunoprecipitates with Cdt1-specific antibodies contained chromatin fragments bearing Cdt1 and geminin (Fig. 4A, right). Thus, the data confirm that Cdt1 and geminin can occur together at the same or closely adjacent sites on chromatin fragments of <0.5 kb.

This result prompted us to map the binding sites of Cdt1 and geminin relative to a previously characterized replication origin in the human genome. This origin is located in the region between the two active human genes PRKDC and MCM4. It includes the site where ORC is bound and where the assembly of a prereplication complex is initiated (29, 34).

To determine the Cdt1 and geminin binding sites in this region, we extracted the DNA from the immunoprecipitates and assayed for the presence of specific DNA sequences by quantitative PCR under the conditions described previously (34). The amounts of precipitated DNA sequences were compared with the amounts of amplifiable DNA in cross-linked chromatin before immunoprecipitations (input) to estimate the enrichment achieved by immunoprecipitation. In both the Cdt1 and the geminin precipitates, we detected an enrichment of DNA sequences (Fig. 4B, Ex2) that were adjacent to, but not identical with, the previously determined ORC binding site in the upstream promoter region (Fig. 4B, UPR).

Next, we were interested to find out whether geminin remained on these sites after Cdt1 had been degraded in the early S phase. We have therefore performed in vivo cross-linking experiments with cells synchronized by a double-thymidine procedure and released into S phase. As shown in Fig. 2, Cdt1 is undetectable in these cells, whereas significant amounts of geminin remain on chromatin. We first analyzed the proteins in the immunoprecipitates by Western blotting and found that geminin-specific antibodies precipitated chromatin with geminin (as expected) and coprecipitated Mcm7 protein but not (or very little) Orc2p (Fig. 5A, left). The controls were Mcm7 antibodies, which precipitated chromatin with Mcm7 and geminin (Fig. 5A, right). Because chromatin had been trimmed to fragments with less than 0.5-kb DNA, we conclude that geminin must be located close to Mcm7 but not to Orc2. Ritzi et al. (27) have noted before that Orc proteins and Mcm proteins are not regularly cross-linked to neighboring sites in 2×T-arrested HeLa cells.

We also determined the enrichment of DNA sequences in the precipitates using quantitative PCR as in Fig. 4. In agreement with earlier results (34), we found that Orc2 antibodies preferentially precipitated chromatin fragments with DNA sequences of the upstream promoter region (Fig. 5B, middle,
Geminin-specific antibodies enriched sequences closely bracketing the Orc2-bearing region in the immunoprecipitated chromatin (Fig. 5B, bottom) extending and supporting the results shown in Fig. 4B. We did not observe any differences between early and late S phase cells (not shown) suggesting that geminin does not move from these sites during S phase.

In summary, when both proteins occur simultaneously in the cells at the G1-to-S phase transition, Cdt1 and geminin are linked to the same chromatin region, and geminin remains at that region later in S phase when Cdt1 has disappeared. In the single case that we have analyzed, the geminin-binding region is closely adjacent to an established ORC binding site.

Role of Geminin in the HeLa Cell Cycle—What could be the function of chromatin-bound geminin? One possibility is that bound geminin may prevent the binding of Cdt1 during an ongoing S phase. This would be necessary under conditions...
when Cdt1 is not fully degraded or when the rate of Cdt1 production exceeds the rate of degradation. Indeed, Cdt1 continues to be synthesized during S phase in HeLa cells. We showed this by releasing 2×T-arrested cells into growth medium containing MG132, a potent inhibitor of proteasome-mediated proteolysis (Fig. 6). No Cdt1 was present in 2×T-arrested cells (see Fig. 6A, 0 h, and Fig. 2, 0 h), but new Cdt1 appeared in increasing amounts after release from the block during a 3-h period in the presence of MG132 (Fig. 6A). Moreover, the newly formed Cdt1 interacted in part with geminin as shown by the coimmunoprecipitation results of Fig. 6B. Thus in principle, chromatin-bound geminin could function as a safeguard against the unscheduled appearance of Cdt1 in S phase.

We note in Fig. 6A that the amounts of soluble Cdt1 relative to bound Cdt1 seemed to be higher in MG132-treated than in untreated cells (see Fig. 1) as if the new Cdt1 associated less readily with chromatin under these conditions. This could mean that some of the normal Cdt1 binding sites were not accessible (perhaps by bound geminin). Alternatively, Cdt1, synthesized in the presence of MG132, could be modified by phosphorylation or ubiquitination resulting in a reduced affinity for DNA (7, 16, 17). Indeed, Cdt1 from MG132-treated cells was found to have a slower than normal electrophoretic migration rate as expected for phosphorylated Cdt1 (Fig. 6C). Regardless, however, some new Cdt1 did bind to chromatin and could attract geminin explaining why more geminin was found on chromatin in MG132-treated than in control cells (Fig. 6C).

Of course, in rapidly proliferating untreated HeLa cells, new Cdt1 is soon degraded in S phase and cannot accumulate. Therefore, if its major function were indeed the prevention of Cdt1 function, geminin should be dispensable in HeLa cells. To test this possibility, we used an RNA interference approach to knock down geminin and found, as predicted, that geminin-depleted HeLa cells proliferated without apparent changes in cell cycle parameters (Fig. 7A) (as also described recently by others (17)) including the loading of Mcm proteins (Fig. 7B).

The purpose of this study was to learn more about the regulatory protein geminin, which has attracted recently much attention because it may be a key factor integrating cell proliferation and cell differentiation (35). More specifically, we were interested in the function of that fraction of geminin that is bound to chromatin in proliferating cells. Here we investigate the behavior of chromatin-bound geminin during the cell cycle.

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### DISCUSSION

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immunofluorescence that geminin is strictly nuclear (13, 24), and we conclude that geminin in the cytosolic fraction has simply leaked from the nuclei during preparation as it has been shown before for numerous other soluble nuclear proteins such as, for example, mammalian Mcm proteins (36).

The established binding partner of geminin is the loading factor Cdt1 (3, 4). In HeLa cells, Cdt1 begins to be synthesized in late mitosis and early G1 phase and is immediately transferred to chromatin in G1 phase. Mammalian Cdt1 has an intrinsic DNA binding activity with no preference for specific DNA sequences (25), and it is likely that Cdt1 is directed to its sites of function by interactions with the stationary ORCs, where it participates in the loading of Mcm proteins. At the end of G1 phase, when all prereplication complexes are completely assembled, Cdt1 becomes dispensable and is inactivated, apparently by two independent processes. One is the phosphorylation of Cdt1 by the cyclin A-dependent kinase and its subsequent ubiquitin-mediated proteolysis (14–17), and the second process could involve geminin. The synthesis of geminin begins with the timely activation of E2F transcription factors and continues during S phase (18, 19). It seems that the new geminin is soon targeted to the chromatin-bound Cdt1 because our communoprecipitations showed that a large fraction of Cdt1 occurs in complexes with geminin (Fig. 2). Moreover, it has been reported that isolated geminin has no intrinsic DNA binding activity (25) and must be tethered to chromatin via a true DNA-binding protein such as Cdt1. Indeed, it has been shown in Xenopus egg extracts that the binding of geminin to chromatin depends on the prior binding of Cdt1 (8). This does not necessarily imply that Cdt1 is the only chromatin-associated interaction partner of geminin. In fact, we noted that geminin-specific antibodies coprecipitated much, but by no means all, Cdt1 (Fig. 2B). This can be in part explained by a dissociation of Cdt1 and geminin during cell fractionation. However, our cross-linking data also suggest that a part of geminin is linked to chromatin fragments that do not simultaneously carry Cdt1. It could be that in this case geminin is connected to a transcription factor as it has been shown for the homebox containing factor Six3 that competes with Cdt1 for binding to geminin (11). Nevertheless, in the single origin region that we have investigated, we identified a geminin binding site in the close vicinity of ORC suggesting that chromatin-bound geminin may have a role in regulating replication initiation particularly because much geminin remains at this binding site after the degradation of Cdt1. Biochemical studies have shown that geminin inhibits the interaction of Cdt1 with Mcm proteins and induces the release of Cdt1 from DNA in vitro (25, 37). This gives no clue as to how geminin is retained on chromatin after Cdt1 release. An interaction with ORC could be an interesting possibility. However, according to our ChIP data (Fig. 6), geminin and ORC do not reside in close proximity, and some mediator proteins must exist to connect geminin to ORC. The underlying molecular mechanisms may have yet to be elucidated.

Geminin at ORC could be involved in blocking the access of Cdt1 to ORC, thereby ensuring that DNA does not overreplicate. This may be important under special conditions or in certain cell types where Cdt1 levels remain high in S phase, but it is not important in HeLa cells where Cdt1 is effectively eliminated. We could indeed show that a depletion of geminin does not lead to an overreplication in HeLa cells.

Although this result may seem plausible in HeLa cells, we must point out that the loss of geminin has different outcomes in different biological systems. Thus, McGarry (9) found that depletion of geminin in early Xenopus embryos causes a proliferation arrest in G2 phase immediately after midblastula transition probably because of an activation of the DNA replication/DNA damage checkpoint pathway. Quinn et al. (39) studying geminin mutants of Drosophila detected overreplication defects in oogenesis and in late embryogenesis. In addition, Mihaylov et al. (40) used an RNAi procedure to down-regulate geminin in Drosophila Schneider D2 cells and detected cessation of mitoses and a partial overreplication of the genome, a phenotype that was in part again dependent on a functioning checkpoint pathway. Very recently, Melixetian et al. (41) described that a depletion of geminin caused a partial overreplication of DNA in certain cultured human cells. Thus, it seems that HeLa cells that rapidly and completely destroy their Cdt1 immediately at the beginning of S phase can well tolerate a loss of geminin, whereas other cells are forced to activate their DNA damage checkpoint pathway to avoid overreplication.

While this paper was under revision, several reports have appeared describing an RNAi approach to investigate the effects of geminin depletion on the cell cycle. Working with HeLa cells, Nishitani et al. (17) found that a depletion of geminin had no obvious effects on cell cycle parameters. However, Melixetian et al. (41) studied human HCT116 carcinoma cells, U2OS osteosarcoma cells, and TIG3 diploid fibroblasts and detected that RNAi-mediated geminin depletion causes a partial rereplication within a cell cycle accompanied by an activation of the ATR/ATM checkpoint pathway (ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia mutated and rad3-related). Significantly, overreplication did not occur when Cdt1 was also repressed. This clearly explains the difference between the cell types investigated. In the HeLa cells that Nishitani et al. (41) and we investigate Cdt1 is not available in S phase because it is rapidly and effectively degraded. Thus, the results of Helin and co-workers (38) support our notion that geminin becomes necessary as a regulatory factor only when the concentration of Cdt1 remain high during S phase. Although Cdt1 is permanently synthesized during the S phase of rapidly proliferating HeLa cells (Fig. 6), it never accumulates, and therefore geminin has no opportunity to function as a negative or positive regulator (protecting Cdt1 from proteasome-mediated degradation, as proposed by Bailabeni et al. (38)). In fact, we find the seemingly conflicting data to be a neat demonstration of the fact that cell physiology cannot always and simply be predicted from the behavior of a single biochemical pathway.

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