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Geminin, an Inhibitor of DNA Replication, Is Degraded during Mitosis

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Summary

We describe a novel 25 kDa protein, geminin, which inhibits DNA replication and is degraded during the mitotic phase of the cell cycle. Geminin has a destruction box sequence and is ubiquitinated anaphase-promoting complex (APC) in vitro. In synchronized HeLa cells, geminin is absent during G1 phase, accumulates during S, G2, and M phases, and disappears at the time of the metaphase–anaphase transition. Geminin inhibits DNA replication by preventing the incorporation of MCM complex into prereplication complex (pre-RC). We propose that geminin inhibits DNA replication during S, G2, and M phases and that geminin destruction at the metaphase–anaphase transition permits replication in the succeeding cell cycle.

Introduction

Many biochemical and cytological events of the cell cycle are coordinated by the proteolysis of key regulatory proteins (King et al., 1996a). The degradation of B-type cyclins has been studied in the most detail. B-type cyclins are activating subunits of the mitotic kinase $p34^{cdc2}$, which is required for entry into mitosis (Nurse, 1990). Mitotic kinase activity does not persist indefinitely because $p34^{cdc2}$ activates a proteolytic system that degrades B-type cyclins. With the loss of the activating subunit, $p34^{cdc2}$ kinase activity drops and the cells return to interphase. The critical importance of cyclin destruction in controlling the exit from mitosis is demonstrated by the effects of nondegradable mutant forms of cyclin B (Glotzer et al., 1991). These mutants retain the ability to activate $p34^{cdc2}$ but are missing the destruction box, a nine–amino acid sequence that is recognized by the cyclin proteolytic system. If one of these mutants is introduced into cells, they become arrested in mitosis with condensed chromatin and high levels of $p34^{cdc2}$ kinase activity.

B-type cyclins are destroyed by ubiquitin-mediated proteolysis (Hochstrasser, 1996). In this pathway, cyclin proteolysis is carried out in two steps. First, a covalently attached polymer of the small protein ubiquitin is built up on the cyclin. Then, a multisubunit protease complex called the proteasome recognizes and destroys the polyubiquitin–cyclin complex. The cyclin is degraded to small peptides, and the polyubiquitin chain is disassembled back to ubiquitin monomers. The attachment of ubiquitin to cyclin occurs in three enzymatic steps. First, ubiquitin-activating enzyme (E1) forms a high-energy covalent bond to ubiquitin at the expense of ATP. Second, the activated ubiquitin is transferred to a second enzyme, ubiquitin-conjugating enzyme (E2). In the case of cyclin B1, at least two different proteins can perform this second step in vitro, Ubc4 and UbcX/E2-C (King et al., 1994). Finally, the ubiquitin is transferred to cyclin by a 1.5 MDa multiprotein complex called the anaphase-promoting complex (APC) or the cyclosome (King et al., 1994; Sudakin et al., 1995). APC is thought to recognize the destruction box and attach ubiquitin to nearby lysine residues (King et al., 1996b). The activity of APC is cell cycle regulated. APC isolated from mitotic cells ubiquitinates cyclin B with a much higher efficiency than APC isolated from interphase cells.

APC displays a remarkable degree of substrate specificity. It promotes the ubiquitination of a small number of proteins, all of which contain a destruction box sequence. For example, APC-mediated destruction of an "anaphase inhibitor" is required to bring about separation of sister chromatids on the mitotic spindle (Holloway et al., 1993; Irniger et al., 1995). The Pds1 protein from budding yeast and Cut2 protein from fission yeast show some of the expected characteristics of this inhibitor (Cohen-Fix et al., 1996; Funabiki et al., 1996). Both proteins contain a destruction box sequence. In both cases, if this sequence is deleted or mutated, the protein is stabilized and the chromosomes fail to separate. APC also ubiquitinates Ase1p, a protein that binds to microtubules and promotes elongation of the spindle and separation of the spindle poles during anaphase (Juang et al., 1997). Ase1p contains several sequences similar to the cyclin B destruction box, and the protein is stabilized if a particular one of these is mutated. The stable form of Ase1 delays disassembly of the spindle after mitosis, leading to damaged spindles in subsequent mitoses.

This paper describes a novel 25 kDa protein, geminin, which is ubiquitinated by APC and mitotically degraded. Geminin contains a destruction box sequence near its amino terminus that is necessary for these reactions. In synchronized HeLa cells, geminin is absent during G1 phase, accumulates during S, G2, and M phases, and is degraded as the cells exit from mitosis. Bacterially expressed geminin dramatically inhibits the initiation of DNA replication in vitro. The protein interferes with the assembly of prereplication complex at a point between the binding of Xcdc6 to chromatin and the binding of MCM complex. When endogenous geminin is removed from cell extracts by immunodepletion, a single complete round of DNA replication occurs. A model is presented for the biological function of geminin in cell cycle control.

Results

Structure of Geminin

A cDNA encoding geminin was initially isolated using a screening procedure designed to identify proteins that were degraded by mitotic *Xenopus laevis* egg extracts but not by interphase egg extracts. The details of this

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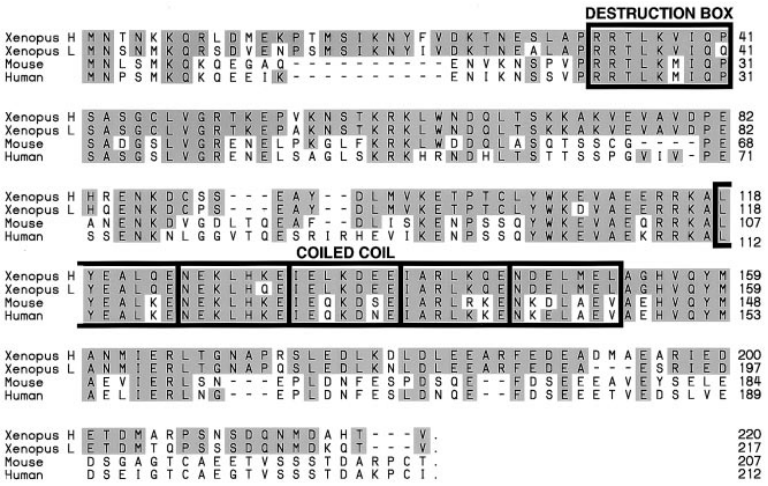

Figure 1. Amino Acid Sequence of Geminin

The predicted amino acid sequences of *Xenopus*, mouse, and human geminin were aligned using the CLUSTAL method. Amino acids identical with the *Xenopus* geminin H sequence are shaded. The destruction box and predicted coiled-coil region are indicated by rectangles. The coiled-coil region was identified using the PARCOIL algorithm (Lupas et al., 1991).

procedure are described elsewhere (Lustig et al., 1997). The screen was very specific; the major proteins identified were B-type cyclins and geminin. A second geminin cDNA was isolated from a screening procedure designed to identify proteins that affected embryonic development (Kroll and Kirschner, submitted). The proteins encoded by the two cDNAs are 89% identical at the amino acid level, and they seem to have identical properties (see below). Because of this close similarity, the protein was named geminin (L. *gemini*, twins). This paper discusses the role of geminin in the cell cycle, and the effects of geminin on embryonic development will be described elsewhere (Kroll and Kirschner, submitted).

The predicted amino acid sequence of the two geminin isoforms is given in Figure 1. The proteins are 219 and 216 amino acids in length and have been designated geminin H and geminin L, respectively. Both proteins have a calculated molecular mass of about 25 kDa, but they migrate aberrantly on polyacrylamide gels with an apparent molecular mass of about 35 kDa (Figure 3A). Neither protein shows any sequence homology to a previously characterized protein. Several structural motifs can be identified by inspection of the amino acid sequence. The amino terminal portion contains a short sequence, RRTLKVIQP, which has homology to the destruction box consensus sequence of mitotic cyclins (RxALGVIxN). This motif begins at amino acid 33, which is roughly the same distance from the amino terminus as the destruction box of mitotic cyclins (Glotzer et al., 1991). The central portion of geminin contains a 35–amino acid sequence from positions 118 to 152, which is predicted to form a coiled-coil domain with five heptad repeats (Lupas et al., 1991). This is a potential site for protein–protein interactions. There are clusters of basic amino acids between positions 50 and 116. These may serve as a nuclear localization signal or as sites of ubiquitin attachment.

Both human and mouse homologs of geminin can be found in the EST databases. However, there is no obvious homologous sequence in the genome of the budding yeast *Saccharomyces cerevisiae*. The amino acid sequences of *Xenopus* geminins H and L, mouse geminin, and human geminin are aligned in Figure 1. Overall, the human and mouse proteins are about 45% identical to the *Xenopus* proteins. The amino acids around the destruction box and the coiled-coil region are more highly conserved (89% and 81% identical) than the amino acids outside these regions. The evolutionary conservation of these sequence motifs suggests that they are important for the protein's biological function.

The experiments in this paper were performed using the cDNA for geminin H, unless otherwise noted. Whenever geminin H and geminin L were compared, they were found to have identical characteristics.

Geminin Is Mitotically Degraded

Xenopus egg extracts were used to demonstrate the mitosis-specific degradation of geminin (Figure 2A). These extracts reproduce in vitro the substrate specificity and cell cycle control of APC activity. B-type cyclins are degraded by mitotic extract but not by interphase extract, and the degradation requires an intact destruction box in the substrate (King et al., 1994; Sudakin et al., 1995).

Geminin cDNA was transcribed and translated in vitro in the presence of [^{35}S]methionine. The radioactive protein was then mixed with either interphase or mitotic egg extract. At various times, an aliquot of the reaction mixture was removed, and the amount of geminin remaining was determined by electrophoresis and quantitative autoradiography. The protein was stable in interphase extract with a half-life greater than 90 min (Figure 2A, top left). In mitotic extract, the protein was unstable and disappeared with a half-life of 15 min (Figure 2A, top middle). Geminin H, geminin L, and cyclin B1 were mitotically degraded with similar kinetics (data not shown).

When the autoradiogram was overexposed, a ladder of high molecular weight bands was detected above the geminin parent band (Figure 2A, bottom). The ladder appeared when geminin was incubated in mitotic extract but not in interphase extract. The bands in the ladder represent geminin conjugated to different numbers of ubiquitin residues. If the extract is supplemented with hexahistidine-tagged ubiquitin, the bands in the ladder acquire the ability to bind to nickel-agarose beads (data

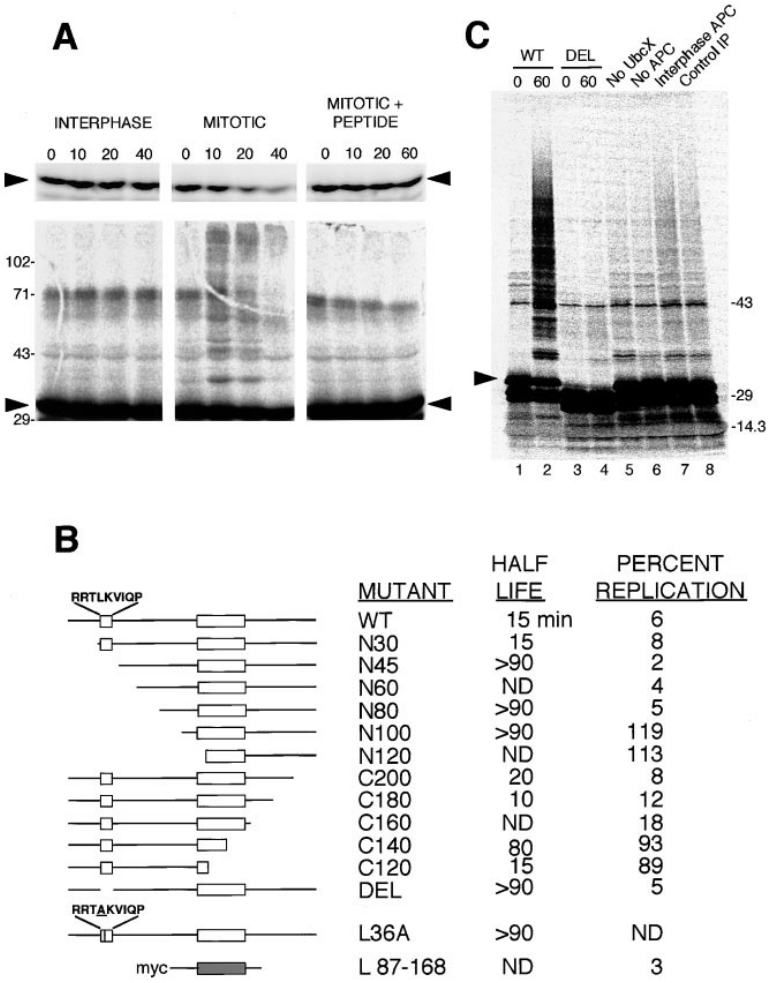

Figure 2. Geminin Is Degraded by the APC Pathway

(A) Geminin is mitotically degraded. [^{35}S]methionine-labeled geminin H was incubated with interphase extract, mitotic extract, or mitotic extract containing 100 μg/ml cyclin B1 D-box peptide. Proteins were detected by electrophoresis and autoradiography.

(B) Domains of geminin H. A series of deletion and point mutations was constructed by site-directed mutagenesis. The small rectangle indicates the destruction sequence RRTLKV IQP, and the large rectangle indicates the predicted coiled-coil region. The name of each mutant indicates whether the deletion is from the N or the C terminus and the amino acid at which the deletion ends. The half-life of each construct in mitotic extract was measured as in (A). The percent of control DNA replication occurring in the presence of each mutant (50–64 μg/ml) is also indicated. The average result for two independent experiments is reported.

(C) Geminin is ubiquitinated by APC in vitro. [^{35}S]methionine-labeled geminin^{WT} or geminin^{DEL} was incubated in a reaction mixture containing ATP, ubiquitin, bacterially expressed proteins E1 and UbcX, and APC immunoprecipitated from mitotic extract with anti-cdc27 serum. The arrowhead indicates full-length geminin^{H}. The two smaller bands represent false initiation by the reticulocyte extract at two internal AUGs. Lanes 1–4: ubiquitin conjugates formed at t = 0 and t = 60 min. Lanes 5–8: same as lane 2, except that various conditions were altered.

not shown). These characteristics of the ladder strongly suggest that geminin is destroyed by ubiquitin-dependent proteolysis.

To confirm that the degradation occurred via the APC pathway, the degradation reaction was repeated in the presence of an unlabeled peptide containing the cyclin B destruction box (Figure 2A, third panel). This peptide is ubiquitinated by APC and inhibits the proteolysis of proteins destroyed by the APC pathway, presumably by a competitive mechanism (Holloway et al., 1993; King et al., 1994). The presence of the cyclin B destruction box peptide inhibited the formation of geminin–ubiquitin conjugates and stabilized the protein in mitotic extracts.

Geminin Contains a Destruction Sequence

In order to see if the RRTLKVIQP sequence constituted a destruction signal, a series of amino and carboxy terminal deletion mutants of geminin was constructed. Figure 2B shows the portion of the coding sequence remaining in each mutant. The positions of the putative destruction box (amino acids 33–41) and the coiled-coil region (amino acids 118–152) are indicated by the small and large rectangles, respectively. The half-life of each mutant protein was measured in interphase and mitotic extract. All the proteins were stable in interphase extract, with half-lives greater than 90 min (data not shown). In mitotic extracts, almost all the mutants with an intact RRTLKVIQP sequence were degraded normally, with half-lives of 10–20 min. The only exception was mutant C140, which may adopt a conformation where the destruction box is inaccessible. For every mutant in which the RRTLKVIQP sequence was deleted, the protein was stable, with a half-life of >90 min. To confirm this result, a small deletion mutant was constructed in which only these nine amino acids were removed (DEL). This protein was completely stable in mitotic extracts. Finally, a point mutation was constructed in which the leucine at position 36 was changed to alanine (L36A). A cyclin B1 mutant that carries the corresponding mutation is completely stable in mitotic extracts (King et al., 1996b). The geminin^{L36A} mutant protein was also stable. These results indicate that the RRT LKVIQP sequence is a required determinant for the destruction of geminin.

Geminin Is Ubiquitinated and Degraded by the APC Pathway

A reconstituted reaction system was used to demonstrate that geminin was directly ubiquitinated by APC (King et al., 1994; Yu et al., 1996). The reaction requires mitotic APC, ubiquitin, the E1 and E2 enzymes, and ATP. Enzymatically active APC can be immunoprecipitated

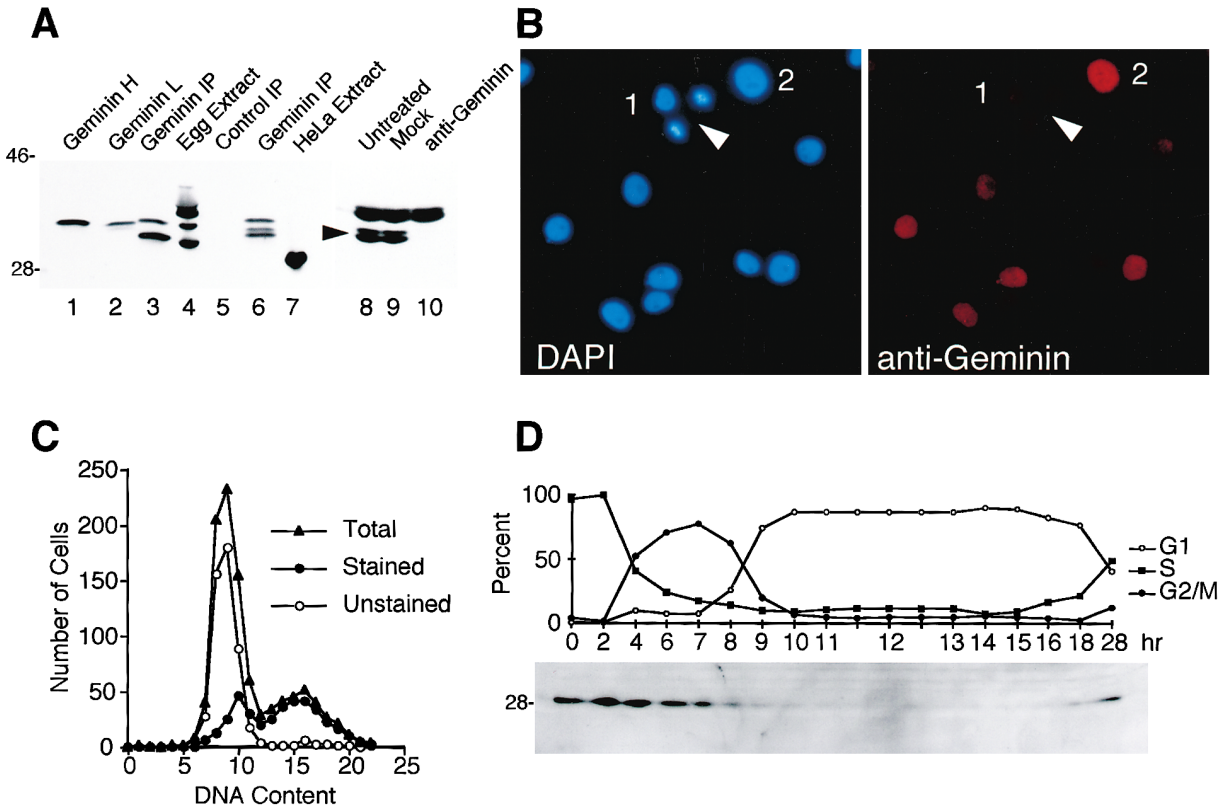

Figure 3. Geminin Is Degraded In Vivo

(A) (Left) Characterization of anti-geminin antibodies by immunoblotting. Lanes 1 and 2, in vitro translated geminin H and geminin L; lanes 3 and 6, proteins immunoprecipitated from extract by anti-geminin antibodies (two independent samples); lane 4, crude egg extract; lane 5, proteins immunoprecipitated by preimmune rabbit IgG; lane 7, crude HeLa cell extract. The positions of the molecular weight standards are indicated on the left. (Right) Immunodepletion of geminin from CSF-arrested egg extracts. Supernates of extracts that had been treated with anti-geminin antibody (lane 10) or rabbit IgG (lane 9) were immunoblotted with anti-geminin antibodies. The arrowhead indicates the doublet of geminin bands. Untreated extract is in lane 8.

(B) Geminin is a nuclear protein. Asynchronous XL177 cells were stained with DAPI and with anti-geminin antibodies. The arrowhead indicates an anaphase cell. The nucleus labeled "2" stains for geminin but the nucleus labeled "1" does not.

(C) Histograms of DNA content for cells staining for geminin (closed circles), cells not staining for geminin (open circles), and all cells (closed triangles).

(D) Cell cycle abundance of geminin. HeLa cells were arrested just after the G1–S transition using a double-thymidine block. At various times after release, the percentage of cells in each cell cycle phase was determined by FACS analysis, and the amount of geminin was determined by immunoblotting.

from extracts with an antibody directed against one of its subunits, the CDC27 protein. APC that has been immunoprecipitated from mitotic egg extracts is much more enzymatically active than APC immunoprecipitated from interphase extracts.

[^{35}S]methionine-labeled geminin was prepared by translation in reticulocyte lysate and incubated in a reaction mixture containing all the required components (Figure 2C). After electrophoresis and autoradiography, a characteristic ladder of ubiquitin conjugates appeared above the starting material (compare lanes 1 and 2). The ladder did not appear if the destruction box mutant geminin^{DEL} was used as the substrate (lanes 3 and 4), if either UbcX or APC was omitted (lanes 5 and 6), if the APC was immunoprecipitated from interphase extracts (lane 7), or if control serum was substituted for the anti-cdc27 serum (lane 8). These results indicate that ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and mitotic APC (E3) are sufficient to ubiquitinate geminin in vitro and that the reaction requires an intact destruction box in the substrate.

Geminin Is a Nuclear Protein

To determine the subcellular localization of geminin, polyclonal antibodies were raised against bacterially expressed geminin H and affinity purified. On immunoblots, the antibodies recognize both geminin H and geminin L that have been translated in vitro (Figure 3A, lanes 1 and 2). The antibodies precipitate two proteins from *Xenopus* egg extract (lane 3). The band with the higher molecular weight comigrates with both geminin H and geminin L. The band with the lower molecular weight has the same peptide map as geminin H and geminin L (data not shown). It may represent a third geminin gene, or a modified form of geminin H or geminin L. In some extracts, the lower band is resolved as two bands (lane 6). On immunoblots of crude egg extract, the antibodies detect four proteins that range in molecular mass from 33 to 40 kDa (lane 4). The two lower bands comigrate with the immunoprecipitated bands. The two higher bands have different peptide maps than geminin H and geminin L; they represent cross-reacting proteins unrelated to geminin (data not shown). Only the three

immunoprecipitated proteins are degraded when APC is activated by adding calcium to unfertilized egg extracts (data not shown). Quantitative immunoblotting showed that the total geminin concentration varied between 1 and 20 nM in different extracts (data not shown). The anti-geminin antibodies detect a protein in human HeLa cells that is slightly smaller than the *Xenopus* geminins, as predicted from the amino acid sequence (lane 7 and Figure 1). This band is not detected using preimmune serum (not shown).

These antibodies were used to perform immunofluorescence staining of cultured *Xenopus* XL177 cells (Figure 3B). The nuclear DNA was counterstained with DAPI. Geminin was predominantly localized to the nucleus. An identical staining pattern was seen in HeLa and 293 cells (data not shown). To exclude the possibility that the signal was coming from the two cross-reacting proteins, geminin H was myc-tagged at the amino terminus and transfected into hamster (BHK) cells. Using an antibody raised against the myc epitope, the transfected geminin protein was localized predominantly to the nucleus (data not shown).

Geminin Is Degraded In Vivo at the Metaphase–Anaphase Transition

In the immunofluorescence studies, two observations suggested that geminin was being degraded by APC in vivo as the cells progressed through the cell cycle. First, anaphase cells did not stain for geminin (Figure 3B, arrowhead), suggesting that endogenous geminin is degraded at the metaphase–anaphase transition when APC is activated. Second, it was noticed that the interphase nuclei did not stain uniformly; some stained brightly while others stained very weakly (Figure 3B, compare 1 and 2). APC activity persists throughout G1 phase and is switched off at the G1–S transition (Amon et al., 1994; Brandeis and Hunt, 1996). The weakly staining nuclei could be in G1 phase, when active APC would destroy geminin. The brightly staining nuclei could be in S or G2 phase, when APC is inactive, allowing geminin to accumulate.

To confirm this hypothesis, XL177 cells in several microscopic fields were digitally photographed and blindly classified as either brightly staining or weakly staining for geminin. Then, the nuclear DNA content of the cells in each population was quantitated by measuring the intensity of the DAPI fluorescence. Figure 3C shows a histogram of DNA content in each of the two populations and for the total population. The total population shows a typical bimodal distribution of DNA content (triangles). The two peaks represent G1 and G2 cells, respectively (2n and 4n DNA content), and the area in between represents cells in S phase. Virtually all the weakly staining cells had a G1 DNA content (open circles), while the vast majority of brightly staining cells had a G2 DNA content (closed circles). The cells with an intermediate DNA content also stained for geminin. These results suggested that geminin was absent from cells during G1, accumulated during S and G2 phases, and then was destroyed before G1 phase of the next cell cycle.

These results were confirmed using synchronized cells. Because of the technical difficulty of synchronizing *Xenopus* cultured cells, HeLa cells were used instead. The cells were arrested at the beginning of S phase with a double-thymidine block and then released. At various times after release, the geminin level was measured by immunoblotting, and the cell cycle stage was determined by FACS analysis (Figure 3D). The FACS data indicate that the cells completed S phase 2–6 hr after release and exited from mitosis 7–9 hr after release. The immunoblot shows that the geminin concentration was high throughout S phase, G2 phase, and most of M phase. The amount of geminin abruptly declined at the time when the cells exited from mitosis and remained low during the following G1 phase. After 18–28 hr, the cells began to reenter S phase and geminin reaccumulated. This is the pattern expected for a protein ubiquitinated and degraded by the APC pathway.

Geminin Inhibits Nuclear Duplication

To determine the biological function of geminin, one of the stable mutants, geminin^{DEL}, was fused to a six-histidine tag, expressed in bacterial cells, and purified to $>$90% homogeneity (data not shown). This protein is stable in mitotic cell extracts because all nine amino acids in the destruction box have been deleted (Figure 2B). The mutation does not seem to inhibit biological activity (see Figures 4 and 5A, below). As a control, GST protein was expressed and purified in the same way. The effect of these purified proteins on cell cycling was determined both in vivo and in vitro.

Either the GST protein or the mutant geminin^{DEL} protein was injected into one cell of a *Xenopus* egg that had divided once (Figure 4, top). In both cases, cleavage progressed normally in the injected half of the embryo. Similar results were obtained if the same concentration of wild-type geminin was injected (data not shown). By contrast, injection of nondegradable cyclin B Δ90 arrested cleavage on the injected side. The fact that the geminin^{DEL}-injected eggs continued cleaving indicates that the protein does not affect either the process of cytokinesis or the sequential activation and inactivation of the p34^{cdc2}/cyclin B kinase. This result was confirmed biochemically by measuring histone H1 kinase activity in a cycling egg extract (data not shown).

Although the geminin^{DEL}-injected embryos continued cleavage, they did not develop normally. They arrested at the blastula stage and never went on to gastrulate (data not shown). To investigate the cause of this arrest, the blastulae were sectioned and the DNA was stained with Hoechst dye (Figure 4, bottom). The embryos were found to have a very striking and obvious defect; the cells produced by cleavage were completely anucleate. Embryos injected with either the same concentration of wild-type geminin or lower concentrations of nondegradable geminin^{DEL} had small, misshapen nuclei (data not shown). By contrast, embryos injected with the GST protein had normal-appearing nuclei in both the interphase and the mitotic configuration (Figure 4, bottom) and developed normally into tadpoles (not shown).

Geminin Inhibits Nuclear DNA Replication

The injection experiments suggested that the nondegradable form of geminin specifically interfered with some step in nuclear duplication. All of these steps can be reproduced in vitro using demembranated sperm

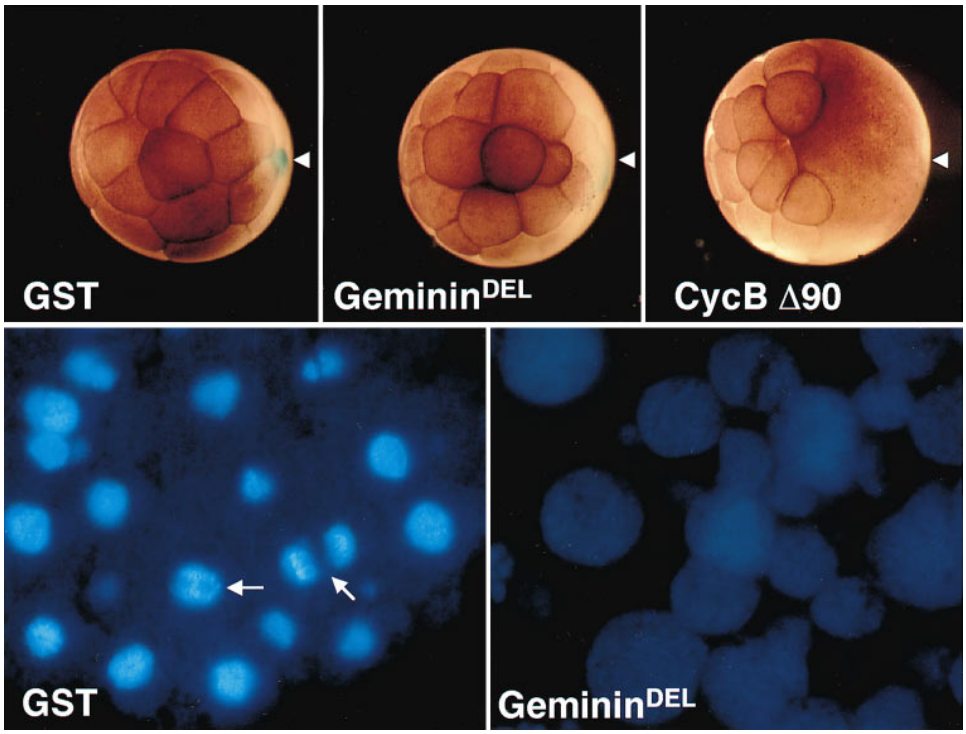

Figure 4. Nondegradable Geminin Inhibits Nuclear Duplication

(Top) One cell of a two-cell *Xenopus* embryo was injected with either GST, nondegradable geminin^{DEL}, or cyclin B Δ90. Photographs were taken 3 hr later. The arrowhead marks the site of injection.

(Bottom) Both cells of a two-cell *Xenopus* embryo were injected with either GST or nondegradable geminin^{DEL}. After 16 hr, the embryos were fixed, sectioned, and stained with Hoechst 33258 dye. Both sections are shown at the same magnification. Two anaphase figures are indicated by arrows.

and extracts made from *Xenopus* eggs. We could not demonstrate any effect of geminin^{DEL} on nuclear assembly, spindle assembly, or chromatid separation (data not shown).

However, geminin strongly inhibited DNA replication. *Xenopus* egg extracts contain all the components needed to replicate sperm DNA. Replication begins about 30 min after an unfertilized egg extract is activated with calcium and continues until all the chromatin has been replicated exactly once (Figure 5B, open circles). In the presence of either geminin^{WT} or nondegradable geminin^{DEL}, there was a profound inhibition of DNA replication (Figure 5A). Very low concentrations of nondegradable geminin^{DEL} protein (20–80 nM) were sufficient to bring about virtually complete inhibition. The exact concentration required to inhibit replication varied from extract to extract. Degradable geminin^{WT} protein was not as potent as geminin^{DEL} at inhibiting replication (Figure 5A, triangles); higher concentrations (300 nM) were needed to produce the same level of inhibition. This difference can be explained by the fact that calcium addition causes a transient burst of APC activity. A sizable fraction of the exogenously added geminin^{WT} protein was degraded after calcium addition, but not the geminin^{DEL} protein (data not shown). Wild-type geminin H and geminin L were equally potent for inhibiting replication (data not shown). GST protein had no inhibitory effect (Figure 5A, open squares).

In order to map the minimal replication inhibitory domain of the geminin H protein, each of the mutants shown in Figure 2B was bacterially expressed, and its effect on the replication reaction was tested. Deletion of up to 80 amino acids from the amino terminal or 60 amino acids from the carboxy terminal did not affect the protein's ability to inhibit replication (Figure 2B). Larger deletions from either end completely destroyed the protein's inhibitory activity. The replication inhibition domain of the protein lies between amino acids 80 and 160, a region that includes the predicted coiled-coil domain. A myc-tagged fragment of geminin L consisting only of amino acids 87–168 (L 87–168) was sufficient to inhibit replication, confirming this result.

Replication Occurs Normally when Geminin Is Immunodepleted

The anti-geminin antibodies were used to immunodeplete geminin from a replication extract and the effects on replication were measured. Immunoblotting showed that >99% of the endogenous geminin could be removed by immunodepletion (Figure 3A, compare lanes 9 and 10). When the endogenous geminin was removed, replication proceeded to the same extent as in control extracts (Figure 5C). Immunodepletion of geminin affected neither the exit from mitosis induced by calcium nor the entry into mitosis induced by cyclin B Δ90 (data not shown).

One possible biological function of geminin would be to inhibit a second round of replication during S, G2, or M phase. To investigate this possibility, density label

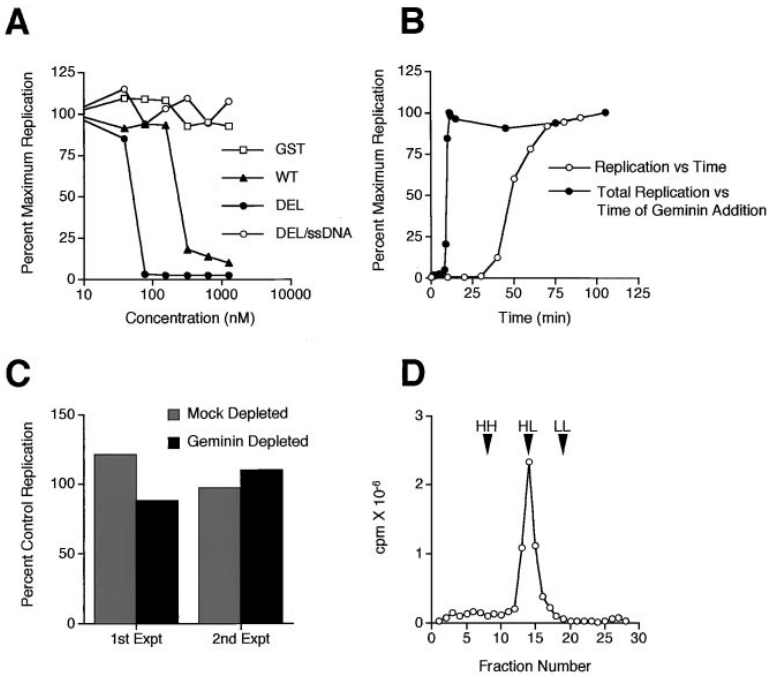

Figure 5. Geminin Inhibits DNA Replication

(A) Replication of sperm head DNA was measured in the presence of GST (open squares), geminin^{WT} (closed triangles), or nondegradable geminin^{DEL} (closed circles) at various concentrations. Replication of single-stranded M13 mp18 DNA was also measured in the presence of geminin^{DEL} (open circles).

(B) Geminin inhibits initiation. Closed circles: a DNA replication reaction was started by adding calcium. At various times after calcium addition, nondegradable geminin^{DEL} was added to an aliquot. The reaction was allowed to proceed for 3 hr, and the total amount of replication was plotted against the time of geminin addition. Open circles: to determine the time when nucleotide incorporation occurred, a separate reaction was done without geminin addition. An aliquot was removed at various times after activation and the amount of replication was measured.

(C) Replication occurs normally when geminin is immunodepleted. In two separate experiments, CSF-arrested extracts were treated with either rabbit IgG or anti-geminin antibody (see Figure 3A, right). Demembranated sperm were added to the supernatants and DNA replication was measured.

(D) A single round of replication occurs in the absence of geminin. Replication was allowed to proceed to completion in a geminin-depleted extract in the presence of BrdUTP and α-[^{32}P]-dATP. The density of the labeled newly replicated DNA was determined by equilibrium centrifugation on a CsCl gradient. The arrowheads indicate the positions of light-light carrier DNA (LL), heavy-light DNA produced by a control reaction (HL), and the predicted position of heavy-heavy DNA (HH).

substitution experiments were performed with geminin-depleted extracts supplemented with BrdUTP (Figure 5D). Replication was allowed to proceed to completion; then, the density of the product DNA was determined by equilibrium centrifugation in a cesium chloride gradient. All of the DNA had a heavy-light density, demonstrating that it had replicated only once. In *Xenopus* egg extracts, the absence of geminin is not sufficient to cause an extra round of DNA replication (see Discussion).

Geminin Inhibits Initiation

Two separate experiments showed that geminin inhibits the initiation of DNA synthesis, but not elongation or ongoing DNA synthesis. First, we found that geminin^{DEL} had no effect on DNA synthesis from a single-stranded bacteriophage M13 DNA template (Figure 5A, open circles). In *Xenopus* extracts, replication from single-stranded templates does not depend on initiation factors and is thought to reflect elongation synthesis only (Harland and Mechali, 1982).

Second, there was a narrow time window during which the replication process was sensitive to geminin. This time window was over long before DNA polymerization began. To demonstrate this sensitive period, a standard replication reaction was started by calcium addition, and stable geminin^{DEL} was added at various times afterward. The reaction was allowed to proceed to completion, and the total extent of replication was determined (Figure 5B, closed circles). If the protein was added within 10 min of calcium addition, replication was completely inhibited. However, if the protein was added at later times, replication proceeded normally. The sensitive period could be compared to the time at which replication began by measuring the amount of α-[^{32}P]-dATP incorporated at various times during a reaction without added geminin (Figure 5B, open circles). Replication did not begin until about 30 min after calcium addition, or about 20 min after the sensitive period had passed. These results suggested that a very early step in the replication process is sensitive to geminin and that elongation synthesis is not affected.

Geminin Inhibits the Formation of Prereplication Complex

Many proteins required for DNA replication are thought to form a prereplication complex (pre-RC) at replication origins (Stillman, 1996; Rowles and Blow, 1997). The pre-RC includes the proteins of origin recognition complex (ORC), cdc6, and a complex of minichromosome maintenance (MCM) proteins. The pre-RC is assembled sequentially. ORC remains directly bound to origins of DNA replication throughout the cell cycle. Shortly before replication begins, first cdc6 and then the MCM complex associate with chromatin. After initiation has occurred, cdc6 and MCM complex are released.

The assembly of the pre-RC can be reconstituted in vitro using *Xenopus* egg extracts and demembranated sperm. After a brief period of assembly, chromatin is pelleted, and the proteins bound to the chromatin are identified by immunoblotting. To see if geminin interfered with the assembly of the pre-RC, different concentrations of the geminin^{DEL} protein were added to these reactions. Figure 6 (bottom) shows that the binding of the MCM complex to chromatin is strongly inhibited by

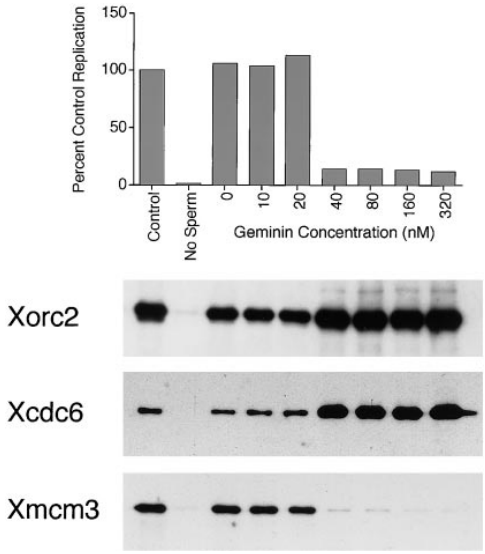

Figure 6. Geminin Disrupts the Assembly of Prereplication Complex

(Top) Geminin was added to a standard replication reaction at different concentrations, and the amount of replication was determined. (Bottom) High-speed supernatant was prepared from the same extract, and geminin was added at various concentrations. After a 30 min incubation, the samples were placed on ice, and the chromatin was pelleted and resuspended in protein sample buffer. Immunoblots were performed using antibodies to Xorc2, Xcdc6, or Xmcm3.

geminin. To compare the concentration needed to inhibit binding and the concentration needed to inhibit replication, standard replication assays were performed using the same extract. Inhibition of MCM binding occurred at the same concentration that inhibited DNA replication (Figure 6, top). By contrast, the binding of the ORC complex to chromatin was not affected by geminin. The binding of Xcdc6 was markedly enhanced by geminin (Figure 6, bottom). Xcdc6 dissociates from chromatin after the MCM complex is bound (Rowles and Blow, 1997). Geminin seems to arrest the replication reaction before MCM complex binds and before Xcdc6 dissociates, so that more of the Xcdc6 protein is found bound to chromatin.

Discussion

In this paper, we describe a novel protein, geminin, which has two important properties related to the cell cycle. First, it is specifically degraded at the time of the metaphase–anaphase transition. Second, it inhibits the initiation of DNA replication. These two properties are associated with two separate domains of the protein. The mitotic degradation depends on a 9–amino acid destruction box sequence located near the amino terminus. The replication inhibition activity resides in an 80–amino acid region that includes a sequence that is predicted to form a coiled-coil structure. The developmental effects of geminin are mediated by a third separate domain (amino acids 40–90) through an unknown mechanism (Kroll and Kirschner, submitted).

Geminin Is Degraded at the Time of Exit from Mitosis

Geminin appears to be ubiquitinated and degraded by the APC pathway that degrades B-type cyclins. Several pieces of evidence support this conclusion. Geminin contains a destruction box sequence near its amino terminus that is recognized by mitotic APC. If this sequence is mutated or deleted, the protein becomes stable in mitotic cell extracts. Geminin is ubiquitinated by purified anaphase-promoting complex (APC) in vitro. This reaction requires an intact destruction box and is carried out efficiently by the mitotic form of APC but not by the interphase form.

Geminin levels fluctuate in cultured cells in the pattern predicted for a protein ubiquitinated by APC. The protein is absent from G1 cells, when APC is active. When APC is turned off at the G1–S transition, geminin accumulates and persists throughout S phase, G2 phase, and most of M phase. Finally, when APC is activated at the metaphase–anaphase transition, geminin levels decline precipitously and remain low throughout G1 phase. In our experiments with synchronized HeLa cells, high concentrations of geminin were present at the arrest point, which was early in S-phase. Geminin probably accumulated during the period of arrest, since at the arrest point the cells would have passed the G1–S transition.

Geminin Inhibits DNA Replication

Low concentrations of bacterially expressed geminin profoundly inhibit nuclear DNA replication. We can demonstrate this effect in vivo, by injecting geminin into embryos, and in vitro, by adding geminin to a DNA replication extract. The protein inhibits replication at extremely low concentrations (20–80 nM). This is comparable to the concentration at which p21 inhibits replication in *Xenopus* extracts (200 nM) or the physiological concentration of known replication proteins like Xcdc6 (80 nM) or Xorc2 (100 nM) (Jackson et al., 1995; Carpenter et al., 1996; Coleman et al., 1996). The bacterially expressed geminin seems less potent than the native protein, since the endogenous geminin concentration in eggs is 1–20 nM. Because geminin is a nuclear protein, the effective concentration at the site of DNA replication may be much higher.

Several pieces of evidence suggest that the endogenous geminin protein inhibits replication under physiological conditions. First, replication occurs normally when the protein is immunodepleted from extracts. Second, when bacterially expressed geminin is added to a concentration sufficient to inhibit replication and then removed by immunodepletion, replication is normal (data not shown). These results indicate that the bacterially expressed protein is not acting in a dominant negative manner by binding and sequestering an essential replication component. Finally, when synthetic geminin RNA is translated in *Xenopus* extracts, the protein produced inhibits replication (data not shown). This excludes the possibility that the bacterially expressed protein has an activity that the endogenous protein lacks due to a difference in modification.

At a molecular level, geminin arrests the sequential assembly of prereplication complex (pre-RC) on DNA at

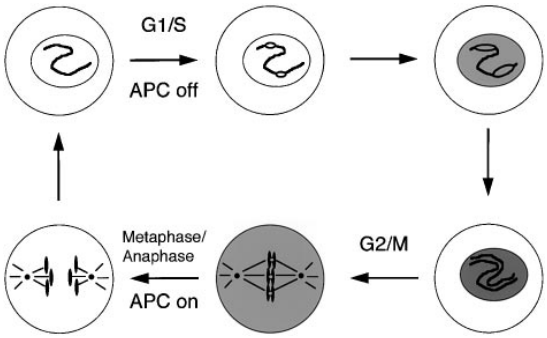

Figure 7. Model for the Biological Function of Geminin

During G1 phase (upper left), APC is active and the geminin concentration is low. At the G1–S transition, APC is inactivated and geminin begins to accumulate (gray shading). After a certain point, enough geminin has accumulated to prohibit further initiation during either late S, G2, or M phase. At the metaphase–anaphase transition, APC activity is restored and geminin is degraded, allowing DNA replication to proceed in the next cell cycle.

origins of replication. The incorporation of MCM complex into pre-RC is strongly inhibited by geminin, while the incorporation of Xcdc6 is enhanced. The arrest in pre-RC assembly occurs between the binding of Xcdc6 and the binding of MCM complex. Xcdc6 is thought to bind to chromatin and assist the recruitment of MCM complex, after which it is released. Our results suggest that if the binding of MCM complex is inhibited, Xcdc6 release does not occur. The MCM complex binds to chromatin 15–30 min after a replication reaction is started (Chong et al., 1995; Kubota et al., 1995), and geminin is able to inhibit replication during the first 10–15 min of the reaction (Figure 5B). This temporal correspondence suggests that the effect of geminin on the binding of MCM complex is rather direct. Apparently geminin does not cause release of MCM complex that is already bound to chromatin.

The exact biochemical mechanism by which geminin inhibits MCM binding is unknown. An 80–amino acid domain of geminin is sufficient to inhibit replication in vitro. Part of this domain is predicted to form a coiled-coil structure, suggesting that it is a site of protein–protein interactions. The simplest model is that geminin binds to and sequesters MCM complex or some other component of pre-RC and prohibits it from participating in complex formation. In preliminary experiments, we have been unable to coimmunoprecipitate geminin with either Xorc2, Xcdc6, or Xmcm3 (data not shown). DNA replication also depends on the activity of certain cyclin-dependent kinases (Stillman, 1996). Some kinases such as Cdk2/cyclin E are required for DNA replication, while others such as cdc2/cyclin B inhibit the process. Geminin may affect the activity of one or more of these kinases. In preliminary experiments, geminin has no measurable effect on immunoprecipitated Cdc2, Cdk2, or cyclin E-associated kinase activity (data not shown).

Model for Geminin's Role in Cell Cycle Control

We present the following model for the biological activity of geminin in cell cycle control (Figure 7). We propose that the function of geminin is to prohibit the initiation of DNA replication at inappropriate times during the cell cycle. The level of geminin protein is controlled primarily by the activity of APC, though there may be additional transcriptional or posttranscriptional mechanisms. During G1 phase, when APC is active, the geminin concentration is too low to inhibit replication. At the G1–S transition, APC is inactivated and geminin begins to accumulate. Initially, the geminin concentration is insufficient to inhibit pre-RC assembly, and DNA replication begins. As S phase progresses, enough geminin accumulates to prevent further assembly of pre-RC. Because geminin has no effect on elongation, DNA replication continues to completion. The protein continues to inhibit replication throughout the remainder of S phase, G2 phase, and most of M phase. At the metaphase–anaphase transition, APC is activated and geminin is abruptly degraded. The degradation of geminin permits another round of replication to begin in the next cell cycle.

The classic cell fusion experiments of Rao and Johnson (Rao and Johnson, 1970) demonstrated the existence of a mechanism that prevents replication after S phase. They showed that when a G1 phase cell and an S phase cell are fused, the G1 nucleus replicates prematurely. This implies that the cytoplasm of the S phase cell contains an activator of replication and that the G1 nucleus is capable of responding to it. However, when a G2 phase cell and an S phase cell are fused, the G2 nucleus does not replicate. Apparently, a G2 nucleus cannot respond to the S phase replication signals. Passage through mitosis must restore the ability of the nucleus to replicate in the next cell cycle. Several models have been proposed to explain the failure of G2 nuclei to undergo replication.

One model proposes that G2 nuclei lack an essential replication component called licensing factor (Blow and Laskey, 1988). Licensing factor is postulated to be present in G1 nuclei and consumed during the replication process. When the nuclear envelope breaks down during mitosis, the nuclear store of licensing factor is replenished from cytoplasmic stores, allowing DNA replication in the next cell cycle. The licensing model is supported by the observation that permeabilization of a G2 nucleus with detergents allows a second round of replication in *Xenopus* extracts (Leno et al., 1992). A protein complex that exhibits many of the expected characteristics of licensing factor has been partially purified (Rowles and Blow, 1997).

Other models propose that G2 nuclei contain an inhibitor of replication that is destroyed at the time of exit from mitosis. Previous work in yeast has strongly implicated mitotic CDKs as inhibitors of replication during late S and G2 phase. In budding yeast, cdc28/clb kinase inhibits replication by inhibiting the activity of cdc6 (Piatti et al., 1996). Transient inhibition of this kinase allows a second round of replication (Dahmann et al., 1995). In fission yeast, reduction of cdc2/cdc13 kinase activity by a variety of means results in repeated rounds of replication (Stern and Nurse, 1996). Other types of inhibitors may inhibit G2 replication in vertebrate cells. For example, the ts41 mutant hamster cell line undergoes multiple rounds of replication without mitosis at the nonpermissive temperature (Handeli and Weintraub, 1992). The mutation is proposed to inactivate a protein that

inhibits replication and promotes mitosis. Another activity that inhibits replication has been identified in extracts from unfertilized *Xenopus* eggs (Mahbubani et al., 1997). This activity is lost at the time of fertilization.

The properties of geminin exactly match the expected characteristics of a G2 replication inhibitor: the protein is present in G2 nuclei but not G1 nuclei, inhibits DNA replication, and is localized to the nucleus. Cell fusion experiments indicate that any proposed inhibitor must be confined to the G2 nucleus, because when G2 and S phase cells are fused, the S phase nucleus replicates DNA normally (Rao and Johnson, 1970). However, the absence of geminin is not sufficient to allow a second round of replication in *Xenopus* extracts (Figure 5D).

The model outlined in Figure 7 does not exclude the possibility of either positively acting licensing factors or additional G2 replication inhibitors. Because even a small amount of excessive replication would have catastrophic long-term consequences, cells may have evolved several mechanisms that operate in parallel or in series to ensure that reinitiation does not occur. Rereplication may be prevented by different mechanisms during different phases of the cell cycle or in different types of cells. A geminin-dependent mechanism may not operate in budding yeast, since they lack a homologous protein. This suggests that the mechanisms that restrict replication may be different in different organisms.

Experimental Procedures

Xenopus Extracts and Embryos

Interphase, mitotic, and CSF-arrested *Xenopus* egg extracts were prepared as previously described (Murray, 1991; King et al., 1994).

Xenopus eggs were collected, fertilized, and injected according to standard procedures (Kay and Peng, 1991). For histological sectioning, embryos were fixed in MEMFA (100 mM MOPS [pH 7.4], 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde). Paraffin-embedded sections were stained with 0.5 μg/ml Hoechst 33258 dye.

Mutant Construction and Protein Stability Measurement

Deletion mutants of geminin H were made by PCR mutagenesis. The mutant genes were inserted into pET29(a) between the NdeI and XhoI sites. The sequences of the primers used to generate each mutant can be supplied on request. The DNA sequence of each mutant was confirmed by dideoxy sequencing. Degradation assays were performed as previously described (King et al., 1996b).

Bacterial Protein Expression

The NdeI-XhoI DNA fragment encoding each geminin mutant was inserted into pET28(a). This manipulation attaches a hexahistidine tag to the 5′ end of the coding sequence. A DNA fragment encoding GST was amplified from pGEX-3X and inserted into the same vector. The hexahistidine-tagged proteins were expressed in *E. coli* strain BL21 and purified according to standard protocols (Qiagen). Proteins were dialyzed against 10 mM HEPES (pH 7.7), 200 mM NaCl before use.

Antibodies

Hexahistidine-tagged wild-type geminin H was used to immunize rabbits. Anti-geminin antibodies were purified by affinity chromatography using the immunogen coupled to CNBr-activated Sepharose 4B beads (Pharmacia) (Harlow and Lane, 1988). Immunoblots were performed according to standard procedures using the antibodies at 1:1000 dilution. For immunofluorescence, tissue culture cells were fixed with 4% formaldehyde in microtubule-stabilizing buffer (MTSB: 80 mM Na-PIPES [pH 6.8], 1 mM $MgCl_2$, 5 mM EGTA). The anti-geminin antibodies were used at a dilution of 1:1000–1:4500, and the secondary antibody (CY3-conjugated donkey anti-rabbit) was used at 1:500.

For immunodepletion of geminin, the affinity-purified antibody was covalently attached to Affi-Prep protein A beads (Bio-Rad) using dimethylpimelimidate (~1 μg antibody/1 μl beads) (Harlow and Lane, 1988). The beads were washed with CSF-XB and added to fresh CSF extract (5 μl beads/100 μl extract). The mixture was rotated at 4°C for 1 hr, and the supernatant was recovered after brief microcentrifugation. A second aliquot of beads was added and the immunodepletion procedure was repeated.

Immunoprecipitations were performed as described previously (Jackson et al., 1995). Geminin was eluted off the beads with 100 mM glycine (pH 2.5), and the eluate was neutralized by adding 1/10 vol of 1 M Tris-HCl (pH 8.0).

DNA Replication Assays

DNA replication was measured using CSF-arrested *Xenopus* egg extracts and demembranated sperm (Leno and Laskey, 1991). Density substitution with BrdUTP and CsCl gradient equilibrium centrifugation were performed as previously described (Blow and Laskey, 1986; Hutchison, 1993).

Chromatin Binding Assays

Two different protocols that gave the same results were used to measure the binding of proteins to chromatin. In the first protocol, demembranated sperm were added to low-speed egg extract, which contains membrane-bound cytoplasmic vesicles (Rowles et al., 1996). After 20 min, the partially assembled nuclei were lysed by dilution into a buffer containing a nonionic detergent, and the chromatin was pelleted through a sucrose cushion. In the second protocol, demembranated sperm were added to high-speed egg extract that did not contain membranes (Hirano and Mitchison, 1993). After allowing chromatin to assemble, sperm DNA and associated proteins were pelleted and resuspended in protein sample buffer. Electrophoresis and immunoblotting were performed using standard procedures.

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GenBank Accession Numbers

The GenBank accession numbers for the four geminin sequences reported in this paper are as follows: *Xenopus* geminin H, AF067856; *Xenopus* geminin L, AF068781; mouse geminin, AF068780; and human geminin, AF067855.