Geminin Has Dimerization, Cdt1-binding, and Destruction Domains That Are Required for Biological Activity*

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Geminin is an unstable regulatory protein that affects both cell division and cell differentiation. Geminin inhibits a second round of DNA synthesis during S and G2 phase by binding the essential replication protein Cdt1. Geminin is also required for entry into mitosis, either by preventing replication abnormalities or by down-regulating the checkpoint kinase Chk1. Geminin overexpression during embryonic development induces ectopic neural tissue, inhibits eye formation, and perturbs the segmental patterning of the embryo. In order to define the structural and functional domains of the geminin protein, we generated over 40 missense and deletion mutations and tested their phenotypes in biological and biochemical assays. We find that geminin self-associates through the coiled-coil domain to form dimers and that dimerization is required for activity. Geminin contains a typical bipartite nuclear localization signal that is also required for its destruction during mitosis. Nondegradeable mutants of geminin interfere with DNA replication in succeeding cell cycles. Geminin's Cdt1-binding domain lies immediately adjacent to the dimerization domain and overlaps it. We constructed two nonbinding mutants in this domain and found that they neither inhibited replication nor permitted entry into mitosis, indicating that this domain is necessary for both activities. We identified several missense mutations in geminin's Cdt1 binding domain that were deficient in their ability to inhibit replication yet were still able to allow mitotic entry, suggesting that these are separate functions of geminin.

Cell division and cell differentiation are tightly coupled during embryonic development. In many ways, cell division and differentiation are mutually exclusive; most differentiated cells in the adult are unable to divide, whereas relatively undifferentiated stem cells continue to divide throughout the life of an organism. Cancerous cells are characterized both by uncontrolled cell division and by a loss of differentiated function. The molecular pathways that link cell division and differentiation are poorly understood.

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Geminin is an unstable 25-kDa protein that has profound effects on both cell division and cell differentiation (1–4). Geminin is a protein of complex multicellular organisms; it is found universally in vertebrates and in Drosophila but is absent from yeasts and the nematode Caenorhabditis elegans. Several different activities of geminin have been described.

Geminin prevents a second round of DNA replication during S and G2 phase by inhibiting the reassembly of prereplication complex, a collection of essential replication factors that assembles on replication origins before DNA synthesis begins (1). Geminin binds and inhibits the protein Cdt1, an essential component of prereplication complex with an unknown biochemical function (5, 6). Geminin is destroyed by ubiquitin-dependent proteolysis during mitosis at the metaphase/anaphase transition, which allows a new round of DNA synthesis in the succeeding cell cycle (1).

Geminin also induces entry into mitosis by antagonizing the checkpoint kinase Chk1. When geminin is depleted from Xenopus embryos or cultured somatic cells, Chk1 accumulates in its active phosphorylated form (7, 8). Chk1 activation leads to phosphorylation and inhibition of the mitotic protein kinase Cdc2. The mechanism by which geminin influences Chk1 activity is unknown. It might affect Chk1 activity indirectly by preventing replication abnormalities, or it may be part of a regulatory pathway that directly down-regulates the kinase.

Several groups have described specific effects of geminin on the development and differentiation of embryonic cells. In early Xenopus embryos, geminin induces uncommitted ectodermal cells to differentiate into nervous tissue (2). The mechanism of this induction is unknown, but the activity is reproduced by a fragment of the protein consisting of amino acids 38–89. More recently, it has been reported that geminin can inhibit eye formation in medaka fish embryos by binding and inhibiting the transcription factor Six3 (3). Geminin can also perturb the axial segmentation pattern of chick embryos by binding and inhibiting transcription factors in the hox gene family (4). These same workers also reported that geminin binds to Smmh1, a protein in the polycomb gene family. The biological consequences of this interaction were not described, but the association suggests that geminin might modify chromatin structure.

It has been difficult to understand how a small 25-kDa protein can have such diverse biological effects. Geminin is not homologous to any previously characterized protein. Sequence analysis indicates that geminin has an internal coiled-coil domain consisting of at least five heptad repeats (Figs. 1B and 10). A nine-amino acid destruction box located near the amino terminus is required for the ubiquitylation and destruction of geminin during mitosis (1). The region between the coiled-coil and the destruction sequence is rich in basic amino acids that could serve as nuclear localization signals or points of ubiquitin

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attachment. The carboxyl terminal region is rich in acidic amino acid residues but otherwise poorly conserved among species.

The purpose of this study was to define the structural and functional organization of the geminin protein. We found that geminin dimerizes through its internal coiled-coil domain with an association constant less than 100 nM. Only the dimerized form of the protein is biologically active. Geminin contains a typical bipartite nuclear localization signal (NLS) in the basic domain close to the destruction box. The binding site for Cdt1 is immediately adjacent to the coiled-coil domain and overlaps it slightly. We generated two non-binding mutants and showed that they are nonfunctional. Finally, we identified several missense mutants in the Cdt1-binding domain that inhibit replication poorly yet still promote entry into mitosis, indicating that these two functions are separable.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Affinity-purified anti-geminin antibodies were raised by immunizing rabbits with full-length Xenopus geminin H (1). His Probe antibodies (sc-804) and agarose-conjugated anti-Myc antibody (9e10) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphospecific anti-phospho-Chk1 (Ser345) antibody was purchased from Cell Signaling Technology. For immunofluorescence studies, anti-Myc antibody (9e10) and Cy3-conjugated goat anti-mouse antibody were purchased from Zymed Laboratories Inc. Anti-Myc immunoblots were performed using polyclonal rabbit anti-human Myc antibody purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Plasmid Construction**—For rescue studies, geminin deletion mutants were constructed by PCR amplification of geminin fragments using pCS2-geminin as a template. pCS2-geminin (150–177) was constructed by amplifying an EcoRI fragment of pCS2-geminin encoding amino acids 1–100 and inserting it into the EcoRI site of pCS2-WT (11). Two extra bases were added during the PCR reaction in order to preserve the correct reading frame. A similar method was used to construct geminin (21–80), geminin (81–100), and geminin (161–177). The sequence of each mutant was confirmed by dideoxy sequencing. pET28-geminin clones were used to express geminin protein in bacteria as described (1). Geminin missense mutants were constructed by the QuikChange site-directed mutagenesis (Stratagene) using pCS2-geminin as a template. pCS2-geminin (150–177) was constructed by amplifying an EcoRI fragment of pCS2-geminin encoding amino acids 1–100 and inserting it into the EcoRI site of pCS2-WT (11). Two extra bases were added during the PCR reaction in order to preserve the correct reading frame. A similar method was used to construct geminin (21–80), geminin (81–100), and geminin (161–177). The sequence of each mutant was confirmed by dideoxy sequencing. pET28-geminin clones were used to express geminin protein in bacteria as described (1).

**Protein Purification**—Hexahistidine-tagged proteins were expressed in bacterial strain BL21 and purified using Ni2+-NTA-agarose according to standard techniques (Qiagen). For analytical ultracentrifugation analysis and importin-binding assays, the hexahistidine tag was removed by thrombin treatment, and contaminants were removed by passing the digest over Ni2+-NTA-agarose. For analytical ultracentrifugation analysis, geminin was further purified by gel filtration for the analyzed data points ranged between 80 nM and 40 μM. Data were fitted to multiple models. The most appropriate model was chosen based upon visual inspection of the residual run patterns and upon the best statistics. 95% confidence intervals were determined by Monte Carlo analysis. A minimum of 6000 Monte Carlo iterations were performed. All data analyses were performed using Ultrasmac version 6.2.

**Binding Assays**—Proteins were either expressed in bacteria or produced by in vitro transcription and translation of plasmid DNA in reticulocyte lysate (Promega TNT system). Reactions typically contained 5 ng to 1.5 μg of recombinant protein or 10–25 μl of reticulocyte lysate. Proteins were mixed in a total volume of 25–50 μl and incubated at room temperature for 1 h. After binding, an aliquot was removed to be used as a loading control. Nickel-NTA or antibody-coated beads (2–5 μl of packed beads containing 1 μg of antibody/μl) were added, and the mixture was tumbled at room temperature for 1 h. The beads were recovered and washed with immunoprecipitation buffer (50 mM β-glycerophosphate, pH 7.4, 5 mM EDTA, 0.1% Triton X-100, 100–500 mM NaCl, and 10 μg/ml each leupeptin, pepstatin, and chymostatin). For importin binding assays, the wash buffer also contained 10% glycerol and 1 M NaCl, and the dithiothreitol was omitted. Proteins were separated on polyacrylamide gels and visualized by immunoblotting. For in vivo binding assays, Xenopus oocytes were injected with 10 ng of anti-geminin morpholin oligonucleotide and 200–400 μg of RNA encoding geminin or Cdt1. Our previous work has shown that this amount of RNA produces a physiological concentration of geminin (7).

**Immunofluorescence**—BHK cells were cultured on coverslips in 1× Dulbecco’s modified Eagle’s medium, 10% calf serum, 10% tryptose phosphate broth. Cells were transiently transfected with plasmids encoding tagged geminin mutants using Lipofectamine (Invitrogen). Twenty-four hours after transfection, cells were fixed with 1× phosphate-buffered saline, 3% formaldehyde, permeabilized with 1× phosphate-buffered saline, 0.1% Triton X-100, and stained with 9e10 anti-Myc antibody (Zymed Laboratories Inc.) and CY3-conjugated goat anti-mouse antibody (Zymed Laboratories Inc.). Nuclei were counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole in phosphate-buffered saline. To visualize nuclei in Xenopus embryos, two-cell embryos were injected with fluorogenic (Amersham Biosciences) and allowed to develop to stage 9. Confocal images were taken at 10 μm intervals and projected onto a single plane.
Geminin self-associates through the coiled-coil domain. A. Myc- and His-tagged geminin were translated in reticulocyte lysate separately (Step separ.) or together (Tagg). Myc-tagged geminin was precipitated with 9e10 anti-Myc antibody, and the precipitate was blotted for the His tag. B, map of geminin deletion mutants used in C and D. C, Myc-tagged geminin deletion mutants were translated together with full-length His-geminin. Myc-geminin was precipitated with 9e10 anti-Myc antibody, and the precipitate was blotted for the His tag. D, stage VI oocytes were injected with RNA encoding various Myc-tagged geminin deletion mutants. After inducing maturation with progesterone, Myc-tagged geminin was precipitated with 9e10 anti-Myc antibody and the precipitate was blotted for geminin. The asterisks indicate Myc-tagged geminin deletion mutants, and the arrows indicate endogenous geminin. IP, immunoprecipitation.

DNA Replication Assays—DNA replication assays were performed using cytostatic factor-arrested Xenopus egg extracts and demembranated sperm DNA template as described (1). Proteins were added at a concentration of 50 ng/μl. The negative controls were no template added and no calcium added. The positive controls were addition and geminin dilution buffer only (10 mM HEPES, pH 7.7, 300 mM NaCl). Percentage replication was normalized to the positive controls. The average of at least two measurements is reported for each protein.

Rescue Assays—Two-cell Xenopus embryos were injected with morpholino anti-geminin oligonucleotide and geminin RNA as described previously (7). For each mutant, a minimum of 18 two-cell embryos were injected on each side (36 injections total). Rescue efficiency was calculated as the percentage of rescue produced by the mutant divided by the percent of rescue produced by wild-type geminin, multiplied by 100%. Injection and scoring were performed blindly to avoid bias in the results.

Degradation Assays—Geminin mutants were transcribed and translated from plasmid DNA using reticulocyte lysate (Promega TNT System) in the presence of [35S]methionine. Translation lysate was mixed with cytostatic factor-arrested Xenopus extract in a 1:4 volume ratio. An aliquot was withdrawn for the t = 0 sample. Degradation was initiated by adding calcium, and a second aliquot was taken after 1 h. The destruction of geminin was visualized by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Geminin Dimerizes through the Coiled-Coil Domain—Computer algorithms predict that the mid-portion of the geminin protein folds into a coiled-coil domain consisting of five or more heptad repeats (Fig. 1B). This observation suggests that geminin self-associates, since coiled-coil domains are frequently sites of homotypic protein-protein interactions. To test if geminin forms oligomers in solution, a plasmid encoding Myc-tagged geminin and a plasmid encoding His-tagged geminin were transcribed and translated together in reticulocyte lysate. When the Myc-tagged geminin was precipitated with anti-Myc antibodies, His-tagged geminin was detected in the precipitate by immunoblotting (Fig. 1A, lane 4). His-tagged geminin was not precipitated by anti-Myc antibodies when translated alone (lane 1). This indicates that the two tagged forms of geminin physically associate with each other. The association is greatly reduced if the proteins are translated separately and then mixed (lane 3), suggesting that oligomers of geminin are relatively stable once formed and do not readily exchange subunits.

To determine whether oligomerization occurs through the coiled-coil domain, the binding experiment was repeated using full-length His-tagged geminin and a series of Myc-tagged geminin deletion mutants (Fig. 1, B and C). Removal of amino-terminal amino acids up to residue 117 did not affect the interaction between the two tagged proteins (Fig. 1C, lanes 1–4); nor did removal of carboxyl terminal amino acids beyond residue 180 (lanes 7–9). However, deletion of residues between positions 117 and 160 completely abolished the association (lanes 5 and 6). This indicates that the self-association domain lies between residues 120 and 160, which almost exactly corresponds to the limits of the coiled-coil (residues 118–152).

To see if geminin oligomerizes in vivo, mature stage VI oocytes were injected with 200 pg of RNA encoding Myc-tagged full-length geminin. Injection of this amount of RNA yields an amount of geminin protein that is similar to the endogenous level (7). The oocytes were then treated with progesterone to induce the translation of both the injected and the endogenous geminin RNA. To see if these two proteins associated, Myc-geminin was precipitated with 9e10 anti-Myc antibody, and the precipitate was blotted with anti-geminin antibody. Both Myc-tagged geminin (asterisk) and endogenous geminin (arrowheads) were detected in the precipitate (Fig. 1D, lane 2). The Myc antibody did not precipitate untagged geminin from un.injected eggs (lane 1). This indicates that geminin forms oligomers in vivo at physiological concentrations. To confirm that
the interaction occurred through the coiled-coil, oocytes were injected with different Myc-tagged geminin deletion mutants. Mutants that included the coiled-coil domain associated with the endogenous geminin (lanes 3–6 and 9), and mutants that encroached upon this region did not bind (lanes 7 and 8). In this experiment, gemininC160, consisting of amino acids 1–160, associates less strongly with Myc-geminin than the wild-type. This suggests that the coiled-coil may extend past residue 160.

To determine the number of geminin subunits in the multimer, we performed sedimentation velocity and sedimentation equilibrium analysis of highly purified bacterially expressed geminin. The velocity analysis indicated that 92% of the protein exhibited a sedimentation coefficient of 2.44 S (Fig. 2B). The equilibrium analysis was performed at several different protein concentrations in order to allow for the possibility that several different geminin species may be present in reversible equilibrium with each other. All experimental observations (18 scans total) could be fit to a model in which a single ideal species is present with a molecular mass of 52.69 ± 0.2 kDa. The variance was extremely low at 2.2 ± 10⁻⁵. A plot of the residuals and overlays for this fit is shown in Fig. 2C. The measured molecular weight is in excellent agreement with that predicted for a geminin dimer based upon the protein sequence (51.31 kDa). Adding additional parameters to account for more than one ideal species (e.g. monomer + dimer or monomer + trimer) did not reduce the variance. We conclude that the data are best described by a single species consisting of geminin dimers. Because no monomer could be detected in the preparation, we estimate that the geminin-geminin association constant is less than 100 nM. Combining the data from the equilibrium and velocity analyses, we estimate that the frictional ratio for geminin (f/f₀) is about 2.2. This value suggests that geminin assumes an elongated shape, consistent with the observation that geminin elutes from a gel filtration column at an apparent molecular weight that is markedly higher than the true molecular weight (Fig. 2A).

Geminin Dimerization Is Required for Activity—We previously reported that the coiled-coil domain is required for geminin to inhibit replication (1). In that study, we found that the two deletion mutants that do not dimerize (gemininC120 and gemininC140) do not inhibit replication and that the C160 mutant, which dimerizes less efficiently, is somewhat reduced in its ability to inhibit replication (Table I and Fig. 4B, black bars). The phenotypes of these mutants indicate that only dimerized geminin protein is active as a replication inhibitor.

To see if dimerization is also required for geminin’s biological activity, we tested the deletion mutants to see if they could rescue the lethal phenotype of geminin-deficient Xenopus embryos using a complementation assay that we developed previously (7). When geminin is depleted from Xenopus embryos with antisense oligonucleotides, the embryonic cells arrest in the G2 phase of the cell cycle after the 13th cell division. The phenotype is visually apparent under the dissecting microscope, because the arrested embryos have larger cells than control embryos (Fig. 3, compare A and B). At the arrest point, the checkpoint kinase Chk1 is found in the phosphorylated active form, which can be demonstrated by immunoblotting using a phosphospecific antibody raised against Chk1 phosphorylated on serine 345 (Ser345, Fig. 4A, lane 2). In contrast, control embryos have little phosphorylated Chk1 (lane 1). The arrest caused by geminin depletion can be rescued by injecting wild-type geminin RNA immediately after the antisense oligo-
nucleotide. The rescuing RNA is mutated at eight “wobble” positions so that it does not hybridize to the antisense oligonucleotide, whereas the amino acid sequence is preserved. Suppression of the G₂ arrest by wild-type geminin is accompanied by a reduction in the amount of Ser⁵⁴⁵-phosphorylated Chk1 down to the levels found in control embryos (Fig. 4A, lane 3). The degree of rescue is quantified by calculating the percentage of rescued cells (Fig. 5, lanes 7–9). Typically, 60–100% of wild-type geminin RNA injections produce a large cell size.

To determine the subcellular localization of these proteins in vitro, the geminin mutants were fused to a Myc tag and expressed in cultured BHK cells. The cells were fixed and stained with anti-Myc antibody to determine the localization of the expressed protein (Fig. 5B). Wild-type geminin is exclusively nuclear in about 75% of the transfected cells and both nuclear and cytoplasmic in about 25% (Fig. 5, B and C). When endogenous geminin is stained in Xenopus tissue culture cells, cytoplasmic geminin is virtually never seen except in metaphase cells (1). The high degree of cytoplasmic localization seen here may be an artifact of overexpression. Geminin⁵⁴⁵, the mutant with a deletion of the first 45 amino acids, shows roughly the same distribution as wild-type geminin. In contrast, geminin¹⁰⁰, which carries a deletion of the first 60 amino acids, accumulates predominantly in the cytoplasm in over 95% of the transfected cells. Less than 5% of the geminin¹⁰⁰-transfected cells showed exclusive nuclear localization. These data confirm the presence of an NLS between amino acids 45 and 60.

Embryos injected with these mutants also showed normal low levels of Chk1 phosphorylation on Ser⁵⁴⁵ (Fig. 4A, lanes 7–9), confirming that a G₂ arrest had not occurred. These results indicate that the carboxyl-terminal amino acids from 160 to 219 are not required for biological activity. In contrast, the geminin¹₂⁰ and geminin¹₄₀ are not able to rescue geminin-deficient embryos at all. Virtually all of the embryos injected with RNA encoding either of these mutants arrest at the 13th cell division, as evidenced by visual inspection of the cells (Fig. 4B, gray bars) and by their high levels of phosphorylated Chk1 (Fig. 3A, lanes 6 and 13). These results indicate that geminin must dimerize in order to suppress Chk1 activity in vivo.

Geminin Has a Bipartite Nuclear Localization Signal—Several independent studies have established that geminin is a nuclear protein (1, 2, 17). The amino-terminal portion of geminin contains several clusters of basic amino acids that could serve as a nuclear localization signal (NLS). To map the NLS, wild-type geminin and several different amino-terminal deletion mutants were tested to see if they bound to the nuclear transport protein importin-α. Each mutant was purified from bacteria and incubated with recombinant His-tagged importin-α. The importin was precipitated with nickel-NTA-agarose beads, and the precipitate was blotted for geminin. Full-length geminin was precipitated by the Ni²⁺-NTA beads when importin was present but not when importin was omitted from the mixture (Fig. 5A, lane 1, compare top and middle panels). Mutants with deletions of the first 30 or 45 amino acids also bound tightly to importin-α (lanes 2 and 3), but mutants with deletions of the first 60 or 80 amino acids did not bind above background (lanes 4 and 5). These results indicate that an importin-α binding site lies between amino acids 45 and 60.

To evaluate the biological significance of geminin degradation, we injected geminin-depleted embryos with RNA encoding geminin¹²⁰, a nondegradable mutant in which the destruction box is deleted. Embryos expressing geminin¹²⁰ had an unusual “roughened” appearance (Fig. 3D). The cells were irregularly sized and had a more uniform pigmentation pattern than normal. To see if this was caused by a replication defect, the embryos were also injected with a fluorescent nucleotide analog to visualize the DNA. We found that geminin¹²⁰-expressing embryos had far fewer nuclei than embryos expressing geminin¹⁰⁰ (Fig. 3, compare E and F). The same appearance is seen when embryos are injected with geminin¹⁰⁰ protein (1). We conclude that geminin must be degraded during mitosis in order to allow replication in the next cell cycle.

**Table 1**

| Mutant | Replication | Rescue | Mutant | Replication | Rescue |
|--------|-------------|--------|--------|-------------|--------|
| Neg CO | (100)       | 0      | NDQ    | 1.1         | 99     |
| WT     | 2.1         | (100)  | LTS    | 1.0         | 100    |
| N30    | 5.6         | 95     | DPE    | 1.0         | 106    |
| N50    | 30          | ENK    | 1.1    | 100         |
| N100   | 124         |      | EAY    | 1.1         | 100    |
| N120   | 145         | <3     | DL     | 1.1         | 104    |
| C120   | 76          | <3     | KE     | 0.4         | 97     |
| C140   | 77          | 4.5    | A111G  | 1.5         | 106    |
| C160   | 8.1         | 81     | Y106F  | 1.9         | 95     |
| C180   | 7.9         | 95     | AYPVR  | 7.2         | 124    |
| C200   | 4.9         | 119    | SS     | 1.2         | 112    |
| C210   | 76          | <3     | AEERR  | 6.3         | 108    |
| LAP    | 95          |       | FFK    | 11.4        | 109    |
| DEL    | 0.2         |       | PTC    | 1.2         | 103    |
| T35A   | 95          |       | KKFEV  | 1.2         | 103    |
| S42A   | 107         |       | YWK    | 1.2         | 103    |
| KRK    | 1.6         |       | RTGG   | 1.2         | 103    |
| RTK/KRK| <0.5        |       | SAPD   | 6.6         | <3     |
| RTK/KRK/KK | 0.8 | D100–117 | 59 | <3 |

Fig. 3. Appearance of normal, G₂-arrested, and rescued embryos. Xenopus embryos were injected at the two-cell stage and allowed to develop to Nieuwkoop stage 9 (21). A, control uninjected embryo; B, G₂-arrested embryo injected with antisense oligonucleotide. Notice the larger cell size. C, rescued embryo injected with antisense oligonucleotide and geminin¹⁰⁰. The arrowhead indicates a patch of unrescued cells bordered by the dotted line. D, “roughened” embryo injected with antisense oligonucleotide and RNA encoding geminin¹²⁰. The arrowhead indicates a patch of unrescued cells bordered by the dotted line. E, nuclei in an embryo rescued with geminin¹⁰⁰ RNA. F, nuclei in an embryo rescued with geminin¹²⁰ RNA.
charged residues separated by a short spacer (18). The underlined basic residues are highly conserved among different species from *Drosophila* to humans. To see if this is the NLS of geminin, two mutants were constructed. In one, the KRK sequence was changed to AAA (gemininKRK), and in the other, all of the basic amino acids were changed to alanines (gemininRTKKRKRK) (Fig. 6A). Both mutants accumulated extensively in the cytoplasm when expressed in BHK cells; in each case, about 80% of the cells showed strong or exclusive cytoplasmic staining. These results indicate that amino acids 50–62 constitute a functional bipartite NLS. We believe that the NLS is bipartite because the gemininRTKKRKRK shows more extensive cytoplasmic localization that gemininKRK and because the gemininN60 protein actually includes the KRK sequence.

All three NLS mutants (gemininN60, gemininKRK, and gemininRTKKRKRK) inhibit DNA replication at the same concentration as gemininWT when added to *Xenopus* egg replication extracts (Table I and data not shown). Either transport into the nucleus is not required to inhibit replication or enough protein finds its way into the nucleus even when the NLS is mutated. To see if nuclear transport is required to suppress Chk1 activation, we expressed gemininKRK and gemininRTKKRKRK in geminin-depleted embryos to see if they could prevent the G2 arrest at the midblastula transition. Surprisingly, embryos injected with either mutant had the same “roughened” appearance as embryos injected with RNA encoding the nondegradable mutant gemininDEL; the cells showed irregular size and pigmentation, and staining of the DNA showed fewer nuclei than normal (not shown). This phenotype led us to suspect that proteins with mutations in the NLS were not degraded properly during mitosis. To confirm this suspicion, 35S-labeled gemininWT and gemininRTKKRKRK were synthesized in reticulocyte lysate and added to metaphase-arrested *Xenopus* egg extracts. Geminin degradation was induced by releasing the extracts from metaphase with calcium, and the stability of the labeled proteins was determined by gel electrophoresis and autoradiography (Fig. 6B). Wild-type geminin is significantly degraded in this assay, but gemininRTKKRKRK is completely stable (compare lanes 1 and 2 with lanes 19 and 20). GemininRTK is degraded normally, and gemininKRK is partially degraded (lanes 15–18).

The results with the NLS mutants led us to examine the possibility that sequences outside the destruction box were required for geminin destruction. We previously showed that a mutant with a deletion of all residues amino-terminal to the

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**Fig. 4.** Dimerization is required for geminin activity. A, lanes 1–3, two-cell *Xenopus* embryos were either left un.injected, injected with geminin antisense oligonucleotide, or injected with antisense oligonucleotide and RNA encoding gemininWT. Embryos were allowed to develop to stage 9, and then the amount of Ser345-phosphorylated Chk1 (top) and geminin (bottom) was determined by immunoblotting. An unrelated protein that cross-reacts with the geminin antibody (CRP) serves as a loading control. (lanes 4–13). The experiment was repeated injecting antisense oligonucleotide and RNA encoding different geminin deletion mutants instead of gemininWT. Black bars, recombinant wild-type and mutant proteins were added to *Xenopus* replication extracts, and the extent of replication of sperm DNA was measured.
destruction box (geminin^{N300}) is degraded normally (1). We constructed several different missense mutants mapping near the destruction box and the NLS are indicated. B, geminin mutants were translated in reticulocyte lysate and added to a metaphase-arrested Xenopus egg extract. Geminin destruction was triggered by adding calcium.

FIG. 5. Geminin has a bipartite NLS. A, top, recombinant geminin mutants were incubated with His-tagged importin α. The importin was precipitated with nickel-NTA-agarose beads and the precipitate was blotted for geminin. Middle, same experiment except importin α was omitted from the binding reaction. Bottom, amount of geminin loaded into each binding reaction. B, HIK cells were transiently transfected with constructs expressing Myc-tagged geminin^{WT} or geminin^{RTK/KRK}. Cells were stained with 9e10 anti-Myc antibody. C, HIK cells were transfected with various geminin mutants, and the percentage of cells showing exclusively nuclear, nuclear + cytoplasmic, or exclusively cytoplasmic staining was counted. For each mutant, the average of at least three independent experiments is shown.

FIG. 6. The NLS is required for geminin degradation. A, sequence of missense and deletion mutations. The destruction box and the NLS are indicated. B, geminin mutants were translated in reticulocyte lysate and added to a metaphase-arrested Xenopus egg extract. Geminin destruction was triggered by adding calcium.

dominin^{SASG} had the same “roughened” appearance as embryos injected with geminin^{DEL} (not shown). This indicates that the entire region from the destruction box to the nuclear localization signal is required for geminin destruction.

The Neuralizing Domain—Kroll et al. (2) reported that a fragment of geminin consisting of amino acids 38–89 is sufficient to induce uncommitted embryonic cells to differentiate as neurons. Our mapping studies show that this fragment con-
sists of the NLS and sequences between the NLS and the dimerization domain. To see if this region is required for geminin’s cell cycle functions, we generated an internal deletion mutant, geminin\textsubscript{H9004}\textsubscript{63–80} that preserves the NLS and the dimerization domain but removes most of the neuralizing domain (Fig. 7A). We also generated several site-directed missense mutants between amino acids 63 and 100, targeting amino acids that are well conserved among species (Fig. 7A). Both the deletion and the missense mutants were able to efficiently rescue the G₂ arrest caused by geminin depletion, and all of the missense mutants were able to inhibit replication when expressed in bacteria and added to replication extracts (Fig. 7, B and C, and Table I). Mutations in the neuralizing domain have no effect on the cell cycle activities of geminin.

Geminin Binds Cdt1 through a Domain Adjacent to the Coiled-coil—We previously reported that a small fragment of geminin consisting of amino acids 87–168 is sufficient to inhibit DNA replication in \textit{Xenopus} extracts (1). Because geminin inhibits replication by binding to Cdt1, the Cdt1-binding site must lie within these limits. To develop an \textit{in vitro} assay for mapping the Cdt1 binding domain, we translated Myc-Cdt1 in reticulocyte lysate and incubated it with purified recombinant His-tagged full-length geminin. When the Myc-Cdt1 was precipitated with 9e10 anti-Myc antibody, the precipitate was found to contain His-tagged geminin (Fig. 8A, lane 2). The H and L isoforms of geminin bound equally well to Cdt1 (compare lanes 2 and 3). No geminin was found in the precipitate when Myc-Cdt1 was omitted from the reaction (lane 1). To pinpoint the Cdt1 binding domain, the binding experiment was repeated using full-length Myc-Cdt1 and a series of recombinant His-tagged geminin deletion mutants. The deletion mutants did not all react equally well with the anti-His antibody, although the same amount of protein was added to the reaction as judged by Coomassie staining (Fig. 8A, compare middle and lower panels). Geminin\textsubscript{N100} and geminin\textsubscript{N120} reacted especially poorly. Taking this variability into account, we found that deletion of up to 100 amino acids from the amino terminus of geminin did not affect the interaction with Cdt1 (lanes 4–8) but that removal of the first 120 amino acids abolished it (lane 9). This indicates that the residues between positions 100 and 120 are required for Cdt1 binding. Successive carboxyl-terminal deletions ending at amino acids 200, 180, 160, and 140 were able to bind Cdt1 (lanes 11–14), but a C-terminal deletion ending at amino acid 120 did not bind (lane 10). This suggests that the residues between positions 120 and 140 are also required for Cdt1 binding. Taken together, these data indicate that the Cdt1 binding domain of geminin lies roughly between amino acids 100 and 140.

To confirm these results \textit{in vivo}, we co-expressed Myc-Cdt1 and various geminin deletion mutants at physiological concentrations in \textit{Xenopus} oocytes. Myc-Cdt1 was precipitated with 9e10 anti-Myc antibody, and the precipitate was blotted for geminin. To eliminate background from endogenous geminin, the oocytes were also injected with anti-geminin oligonucleotide (Fig. 8A, bottom, compare lanes 1 and 2). The injected geminin RNAs were “wobbled” so that they would not react with the antisense oligonucleotide (lanes 5 and 6). The anti-Myc antibody efficiently precipitated wild-type geminin when it was co-ex-

**Fig. 7.** Mutations in the neuralizing domain do not affect the cell cycle. \(A\), map of mutations in geminin’s neuralizing domain. \(B\), two cell embryos were injected with anti-geminin oligonucleotide and each of the missense mutants in \(A\). Embryos were allowed to develop to stage 9, and the amount of S345 phosphorylated Chk1 (top) and geminin (bottom) was determined by immunoblotting. An unrelated protein that cross-reacts with the geminin antibody (CRP) serves as a loading control. \(C\), gray bars, the percentage of rescued embryos produced by injection of each mutant. Black bars, recombinant wild-type and mutant proteins were added to \textit{Xenopus} replication extracts, and the extent of replication of sperm DNA was measured. AS, anti-geminin oligo only; ND, not done.
pressed with Myc-Cdt1 but not when Cdt1 was omitted (Fig. 8C, compare lanes 5 and 6). Geminin mutants with deletions of up to 100 amino acids from the amino terminus also bound Myc-Cdt1 (lanes 7–11). GemininN120 could not be scored because it is either poorly expressed or does not react well with the anti-geminin antibody (lane 12). Carboxyl-terminal deletion mutants that truncated the protein beyond amino acid 140 bound to Myc-Cdt1 (lanes 14–18), but the deletion ending at amino acid 120 did not bind (lane 13). In summary, the in vivo experiment confirms that the Cdt1 binding domain lies between residues 100 and 140.

We next sought to generate missense mutants that were compromised in their ability to bind Cdt1. We concentrated our efforts on amino acids 100–117, since this portion of the Cdt1 binding domain lies completely outside the dimerization domain. Deletion of this entire region destroys the geminin-Cdt1 interaction in oocytes (Fig. 8C, lane 18). We first constructed three site-directed mutations that targeted amino acids that are highly conserved among geminin orthologs from different species (geminin^{PTC}, geminin^{YWK}, and geminin^{AKERR}). We found that all three of these mutants bind Cdt1 normally in the oocyte assay (Fig. 9B, lanes 11, 13, and 15). To make random missense mutations in the Cdt1 binding domain, we synthesized a degenerate oligonucleotide encoding amino acids 100–117 in which 10% of the bases were randomly mutated. The oligonucleotide was made double-stranded and used to replace the recombinant His-tagged geminin protein. A cross-reacting protein (CRP) serves as a loading control.

(Fig. 8. Geminin’s Cdt1 binding site is adjacent to the coiled-coil. A, Myc-tagged Cdt1 was translated in reticulocyte lysate and added to each of a series of recombinant His-tagged geminin proteins. Top, Myc-Cdt1 was precipitated with 9e10 anti-Myc antibody, and the precipitate was blotted for the His tag. Middle, recombinant His-tagged geminin mutants were visualized by blotting with anti-His antibody. Bottom, map of the geminin mutants analyzed in this experiment. C, stage VI oocytes were injected with antisense geminin oligonucleotide and RNAs encoding Myc-Cdt1 and an antisense-resistant geminin mutant. After maturation was induced with progesterone, Myc-Cdt1 was precipitated with 9e10 antibody, and the precipitate was blotted for geminin (top panel). The boxed area was exposed to film longer, because not all mutants react equally well with the anti-geminin antibody. The bottom panel shows the total amount of geminin translated. A cross-reacting protein (CRP) serves as a loading control.)
of geminin to Cdt1 is necessary but not sufficient for geminin to inhibit replication. Because the YWK mutation changes tyrosine 106 to an alanine, we considered the possibility that geminin must be phosphorylated on this tyrosine in order to be active. To test this, we constructed gemininY106F, which changes tyrosine 106 to phenylalanine. GemininY106F, however, was able to both inhibit DNA replication and prevent the G2 arrest just like gemininWT (Table I and Fig. 9D).

Although they did not inhibit replication well, gemininYWK and gemininKKFEV were able to rescue the lethal phenotype of geminin-depleted embryos to some extent. The background of rescue assay is so low (~5%) that even partial rescue is significant. The phenotype of gemininKKFEV and gemininYWK mutants indicates that the replication inhibition and the mitotic entry functions of geminin can be separately mutated, suggesting that they represent two different activities of the protein.

**DISCUSSION**

Geminin is an unstable regulatory protein that has several different biological activities. First, it limits the extent of DNA replication to one round per cell cycle by binding and inhibiting the essential replication factor Cdt1 (1, 5, 6). Second, geminin is required for passage from G2 to M phase either by preventing replication abnormalities or by directly down-regulating the checkpoint kinase Chk1 (7, 8). Third, geminin affects cell differentiation during embryonic development by a variety of proposed mechanisms, including inhibition of homeobox-containing transcription factors and possibly effects on chromatin structure (2–4). It has been difficult to understand how one small protein can have such diverse actions.

In this paper, we describe the structural and functional domains of the geminin protein (Fig. 10). We find that geminin forms homodimers in solution and that dimerization is required for activity. The Cdt1 binding domain is located immediately amino-terminal to the coiled-coil and overlaps partially. Geminin mutants that do not bind Cdt1 are nonfunctional. Geminin contains a typical bipartite nuclear localization signal in its basic region. The amino-terminal portion of geminin contains the sequences required for its mitosis-specific destruction. The destruction signal includes the nine-amino acid destruction box and sequences immediately downstream. (2–4). It has been difficult to understand how one small protein can have such diverse actions.

In this paper, we describe the structural and functional domains of the geminin protein (Fig. 10). We find that geminin forms homodimers in solution and that dimerization occurs through the coiled-coil domain. Geminin dimerization is required for activity. The Cdt1 binding domain is located immediately amino-terminal to the coiled-coil and overlaps partially. Geminin mutants that do not bind Cdt1 are nonfunctional. Geminin contains a typical bipartite nuclear localization signal in its basic region. The amino-terminal portion of geminin contains the sequences required for its mitosis-specific destruction. The destruction signal includes the nine-amino acid destruction box and sequences immediately downstream.
C-terminal to it. Interestingly, the nuclear localization signal is required for the geminin destruction. Embryos that express a nondegradable mutant are severely deficient in DNA replication, indicating that geminin destruction is necessary to allow replication after mitosis is complete. Complementation of the lethal G2 arrest of geminin-deficient embryo requires all of the identified domains.

Previous studies have identified the domains of geminin that are required for its developmental effects. A fragment of geminin consisting of amino acids 38–89 is sufficient to induce neural tissue in *Xenopus* embryos (2). Our mapping studies show that this fragment consists of part of the destruction signal, the nuclear localization signal, and sequences between the NLS and the Cdt1 binding domain. We find that the sequences between the NLS and the Cdt1 binding domain are not required for inhibition of replication or for rescue of a geminin-deficient embryo. The mechanism by which geminin induces neural tissue is unknown; our results suggest that the induction is not secondary to an effect on the cell cycle. Geminin structurally resembles transcription factors in the basic HLH leucine zipper class; it has a dimerization domain, an N-terminal basic region, and a C-terminal tail rich in acidic amino acids. We have found, however, no evidence that geminin activates transcription. The acidic tail and most of the basic region can be deleted without affecting the protein’s cell cycle activities. Moreover, we find that geminin cannot activate transcription of a luciferase reporter gene when expressed in NIH3T3 cells (not shown). Two groups have recently reported that geminin affects eye development and embryonic segmentation by binding and inhibiting transcription factors such as Six3 and Hox family members (3, 4). Both groups find that transcription factors compete with Cdt1 for binding to geminin, so presumably both bind to the same site.

Previous observations suggested that geminin forms higher molecular weight multimers in solution. When *Xenopus* egg extract is fractionated on a gel filtration column, geminin elutes in a broad peak with an apparent molecular mass of about 250 kDa (19). Bacterially expressed geminin elutes at about the same position on gel filtration columns (Fig. 2A), yet when its molecular weight is experimentally determined by analytical ultracentrifugation, it is found to be mostly composed of dimers. Geminin’s high frictional coefficient in velocity sedimentation experiments suggests that it has an elongated shape, which may explain its aberrant behavior on gel filtration columns.

Geminin has a classic bipartite NLS consisting of two clusters of basic residues separated by a 7-amino acid spacer (RT-KepvkstKRK). The underlined arginine and the KRK sequence have been strongly conserved among vertebrates and *Drosophila*. Although mutation of the NLS causes geminin to accumulate in the cytoplasm, a significant number of cells still show some nuclear geminin. This may be an artifact of overexpression; geminin is near the size exclusion limit for nuclear pores and may passively diffuse inside. Alternatively, geminin might “piggyback” inside while attached to another protein such as Cdt1. A second conserved cluster of basic amino acids (RRK) is found within the Cdt1 binding domain. We find, however, that a mutant in which two of these residues are changed to alanines (gemininAEERR) (Fig. 9A) has normal nuclear localization (not shown). It has been reported that geminin must be transported into the nucleus in order to be activated as a replication inhibitor (19). We might expect gemininAEERR or gemininRTGKRK to inhibit replication less efficiently than the wild-type protein, because these mutants show defective nuclear import. We cannot, however, demonstrate any such difference; the two mutants inhibit replication at the same concentration as gemininWT (data not shown). In *Xenopus* extracts, a large fraction of endogenous geminin is bound to Cdt1 before nuclear assembly occurs, but very little is bound after assembly (19). Perhaps only unbound geminin is active as a replication inhibitor, and the effect of nuclear assembly is to free geminin from Cdt1.

We find that the destruction signal required for geminin’s mitotic degradation is larger than the canonical destruction box and includes the nuclear localization signal. One explanation for this result is that geminin must be transported into the nucleus in order to be degraded. We disfavor this hypothesis, however, because geminin is degraded in egg extracts even when no nuclei are present (Fig. 5B). Furthermore, we cannot demonstrate any difference in the degradation rate of geminin in extracts containing different concentrations of nuclei (data not shown). It is also possible that gemininN60-AEERR is stabilized because lysines required for covalent ubiquitin attachment are lost. An exhaustive analysis of cyclin destruction, however, showed that there was no lysine or group of lysines that were absolutely required for ubiquitylation of this protein (20).

We previously showed that a fragment of geminin consisting of amino acids 87–160 is sufficient to inhibit DNA replication in *Xenopus* egg extracts (1). This region is here shown to consist of the Cdt1 binding domain and the dimerization domain. Although these two domains overlap slightly, they are clearly distinct; three mutants that do not bind Cdt1 lie completely outside the dimerization domain (gemininSAFP, gemininSAFM, and gemininC140), and the deletion mutant geminin does not dimerize yet still binds Cdt1.

Our results have implications for the mechanism by which geminin inhibits Cdt1. Four mutants that failed to bind Cdt1 (gemininC140, gemininN129, gemininSAFP, and gemininN100–117) also did not inhibit replication (Table II), confirming that geminin affects replication by binding and inhibiting Cdt1. We isolated several mutants that bound Cdt1 yet inhibited replication poorly or not at all (gemininC140, gemininAEERR, gemininFFK, gemininYWK, and gemininY106F), indicating that Cdt1 binding is necessary but not sufficient for this activity (Table II). These results argue strongly that geminin does not simply bind and sequester Cdt1. GemininC140 does not dimerize, indicating that dimerization is also required for replication inhibition. The dimerization domain may recruit a third protein to the geminin-Cdt1 complex, or it may occlude an active site on Cdt1. GemininYWK both dimerizes and binds Cdt1 yet does not inhibit replication at all. We postulate that the highly conserved YWK sequence blocks an active site on Cdt1.

Two models have been advanced to explain why geminin-depleted cells arrest in G2 phase. One model hypothesizes that DNA replication in the absence of geminin causes overreplication of the DNA, which leads to a G2 arrest through Chk1

| Mutant | Cdt1 binding | Inhibition of replication | Rescue of deficiency |
|--------|--------------|--------------------------|---------------------|
| WT     | ++           | +                        | ++                  |
| A111G  | ++           | ++                       | ++                  |
| Y106F  | ++           | +                        | ++                  |
| AYPYF  | ++           | +                        | ++                  |
| SS     | ++           | +                        | ++                  |
| AEERR  | ++           | +                        | ++                  |
| FFK    | ++           | +                        | ++                  |
| PTC    | ++           | +                        | ++                  |
| KKFEV  | +            | +                        | ++                  |
| YWK    | +            | +                        | ++                  |
| C140   | +            | --                       | --                  |
| RTGG   | +            | --                       | --                  |
| SAPD   | --           | --                       | --                  |
| A100–117 | --    | --                      | --                  |
activation. We have identified three mutants (geminin\textsuperscript{YWK} and geminin\textsuperscript{KKFEV}) that are able to prevent a G\textsubscript{2} arrest in Xenopus embryos although they have a reduced ability to inhibit replication (Table II). These results suggest that geminin is more directly required to down-regulate Chk1 that is constitutively activated during S phase, perhaps by binding a second protein. This hypothetical protein may bind to the same site as Cdt1, since this domain of geminin is required to suppress the Chk1-induced G\textsubscript{2} arrest.

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