Geminin Is Targeted for Repression by the Retinoblastoma Tumor Suppressor Pathway through Intragenic E2F Sites*

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The geminin protein is a critical regulator of DNA replication. It functions to control replication fidelity by blocking the assembly of prereplication complexes in the S and G2 phases of the cell cycle. Geminin protein levels, which are low in G0/G1 and increase at the G1/S transition, are controlled through coordinate transcriptional and proteolytic regulation. Here we show that geminin is regulated transcriptionally by the retinoblastoma tumor suppressor (RB)/E2F pathway. Initially, we observed that the activation of RB led to the repression of geminin transcription. Conversely, Rb-null mouse embryonic fibroblasts have enhanced the expression of geminin relative to wild type mouse embryonic fibroblasts. Similarly, an acute loss of Rb in mouse adult fibroblasts deregulated geminin RNA and protein levels. To delineate the responsible regulatory motifs, luciferase reporter constructs containing fragments of the geminin promoter were generated. An analysis of the critical regulatory cis-acting elements in the geminin promoter indicated that intragenic E2F sites downstream of the first exon were responsible for RB-mediated repression of geminin. The direct analysis of the endogenous geminin promoter revealed that these intragenic E2F sites are occupied by E2F proteins, and the mutation of these sites eliminates responsiveness to RB. Together, these data link the expression of geminin to the RB/E2F pathway and represent the first promoter analysis of this important regulator of DNA replication.

Geminin was identified originally as a protein that is degraded by mitotic Xenopus egg extracts, but not by interphase extracts (1), and concurrently during a screen to identify proteins that affect Xenopus development (2). Geminin is a small (25 kDa) protein expressed during the S and G2 phases of the cell cycle but degraded in M phase at the metaphase/anaphase transition via the anaphase-promoting complex-mediated ubiquitination (3, 4). Functional analyses demonstrated that geminin acts to prevent the relicensing of replication origins after they have fired once. This is accomplished by binding to CDT1 (5), a requisite factor for loading MCMs into the prereplication complex. Beginning at the G1/S transition, geminin protein levels accumulate and become sufficient to inhibit CDT1 activity. As soon as the origins of replication have fired once in the S phase, this inhibition of CDT1 prevents the reloading of MCMs onto chromatin until the completion of mitosis when geminin is degraded. Although the levels of geminin mRNA have been shown to increase 2–3-fold at the G1/S transition (6), the mechanism of this transcriptional regulation remains to be elucidated.

RB has several emerging roles in the control of diverse processes outside of the G1/S transition. These include DNA repair, cell death, and DNA replication (7–10). In the case of DNA replication, the activation of the RB pathway results in the repression of numerous target genes. Classically, this repression is achieved through assembling repressor complexes at the E2F family of transcription factor binding sites (11–13). For example, the known targets of RB-mediated repression include several genes that encode components of the MCM complex, which are required for the initiation of DNA synthesis (14–16). DNA polymerase α and PCNA, both required for DNA replication to proceed, also are repressed via the activation of RB (14–16). As the noted repression factors all stimulate DNA replication, their negative regulation is consistent with the action of RB in the inhibition of the S phase. However, in a recent microarray screen, we identified geminin as a putative target for RB-mediated repression. This finding is difficult to reconcile with the classical cell cycle inhibitory role of RB. If geminin in fact is repressed via RB, this would represent a novel function because it would indicate that RB plays an important role not only in inhibiting replication but also in enabling replication to commence. In this study, we observed the repression of endogenous geminin by active RB as well as deregulation of geminin expression with a loss of RB signaling. A comparison of the geminin genes in mouse and rat revealed conserved E2F sites. We cloned the geminin promoter and identified critical regulatory elements. An analysis of the cis-acting elements by reporter assays showed that the geminin promoter is repressed via active RB functioning through intragenic E2F sites.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The Rat-16, A2-4, and A5-1 cell lines were derived and cultured as described previously (17). A2-4 and A5-1 express a phosphorylation site mutant (PSM) of the RB large pocket domain. PSM-RB, in a tetracycline-off-inducible manner. PSM-RB is unable to be phosphorylated by CDK/cyclin activity and is active constitutively. Twenty-four hours prior to harvest, these cultures were washed extensively with phosphate-buffered saline to remove doxycycline (dox) from the medium. The S phase synchronization was performed by culture in aphidicolin as described previously (17). The RB exon 3loxP/loxP mice were obtained from Dr. Tyler Jacks and have been described previously (18). Mouse adult fibroblasts isolated were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% 

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The abbreviations used are: MCM, minichromosome maintenance; RB, the retinoblastoma tumor suppressor; PCNA, proliferating cell nuclear antigen; GFP, green fluorescent protein; PSM, phosphorylation-site mutant; MEF, mouse embryonic fibroblast; CDK4, cyclin-dependent kinase 4; Gmnn, geminin; dox, doxycycline; TraFac, transcription factor binding site comparison.
heat-inactivated fetal bovine serum, glutamine (200 μg/ml), penicillin, and streptomycin (100 μg/ml). Mouse embryonic fibroblasts (MEFs) isolated from Rb+/− and Rb−/− mice were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, glutamine (200 μg/ml), penicillin, and streptomycin (100 μg/ml).

Adenoviral Infections—Cells were infected with p16INK4A- or GFP-encoding adenovirus as described previously (16). Adenovirus encoding cre-recombinase was obtained from the University of Iowa Vector Core.

Microarray Analysis—Total RNA harvested from cell cultures was utilized for microarray studies as described previously using Affymetrix GeneChips U34A, B, and C. RNA from synchronized cells was used to probe Affymetrix GeneChips U34A, B, and C. Geminin is repressed with the induction of PSM-RB. Cell lysates were immunoblotted for geminin and CDK4 as a loading control.

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RT-PCR—RNA was harvested from cell cultures by phenol-chloroform extraction. The following primers were utilized for microarray studies as described previously using Affymetrix GeneChips U34A, B, and C. RNA from synchronized cells was used to probe Affymetrix GeneChips U34A, B, and C. Geminin is repressed with the induction of PSM-RB. Cell lysates were immunoblotted for geminin and CDK4 as a loading control.

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Expression analysis was performed as described under “Experimental Procedures.” C. Cells were infected with p16INK4A-encoding adenovirus for 24 h.

FIG. 1. Geminin is repressed transcriptionally by active RB. A, Rat16, A2-4, and A5-1 cells were grown in the presence or absence of doxycycline for 24 h. Cell lysates were immunoblotted for RB and CDK4 as a loading control. The expression of PSM-RB is induced strongly in the absence of dox. B, Affymetrix GeneChips U34A, B, and C were probed using cRNA generated from the cell lines described previously. Rat16, A2-4, and A5-1 were grown in the presence or absence of doxycycline for 24 h prior to analysis. Geminin was repressed strongly following the induction of PSM-RB. Error bars indicate the mean ± S.E. between samples. C. RNA was harvested from A5-1 cells at time points between 0 and 24 h after the removal of doxycycline from the medium. RT-PCR was performed as described under “Experimental Procedures.” C. Cells were infected with p16INK4A-encoding adenovirus for 24 h.

FIG. 2. Repression of geminin is not dependent on cell cycle position. A, A2-4 and A5-1 cells were grown on 6-well plates. They were treated for 24 h with doxycycline or with doxycycline for 24 h. B, Cells were grown on 6-well plates. They were treated for 24 h with doxycycline or with doxycycline for 24 h.

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the mean and CDK4 in wild type and Rb in both systems. Geminin levels increased with the loss of probe Affymetrix U74Av2 GeneChips.

standard Affymetrix protocols and used to 144h( or cre-encoding adenovirus for 72 and CDK4 in mouse embryonic fibroblasts.

Rb embryonic fibroblasts (Rb) and from mouse vested from wild type and Rb isolated from mouse

Geminin Is Repressed Transcriptionally by RB Activation—We have developed cells lines that inducibly express specific Rb alleles under the regulation of tetracycline (17). As shown in Fig. 1A, the A5-1 and A2-4 cell lines express PSM-RB when the tetracycline analog dox is removed from the medium. This allele is constitutively active and is capable of inducing cell cycle arrest and transcriptional repression. Microarray analyses showed that geminin was a target of RB-mediated RNA attenuation (Fig. 1B). Similar repression was observed when the RB pathway was activated through the expression of p16INK4a, which leads to the dephosphorylation/activation of the endogenous RB (data not shown).

To verify the results of the microarray, we used the RT-PCR analysis. Specifically, A5-1 cells were grown in the presence or absence of dox for up to 24 h. Recovered RNAs were reverse-transcribed and amplified for geminin or glyceraldehyde-3-phosphate dehydrogenase as a control. Under these conditions, the induction of PSM-RB lead to the attenuation of geminin RNA levels with rapid kinetics (Fig. 1C). If the effect of RB on geminin is of functional significance, the protein levels also should be attenuated. By immunoblot analysis, no change in the geminin expression was observed in the parental Rat-16
cells (Fig. 1D, lanes 1 and 2) but geminin protein levels were attenuated in the presence of PSM-RB in A5-1 cells (Fig. 1D, lanes 3 and 4). Similarly, the ectopic expression of p16INK4A resulted in significant attenuation of geminin protein levels (Fig. 1D, lanes 5 and 6). Thus, the activation of the RB pathway either through constitutively active alleles of RB or the action of p16INK4A resulted in the attenuation of geminin RNA and protein levels.

In principal, the attenuation of geminin could be attributed to indirect effects of RB on cell cycle position. To eliminate changes in cell cycle position, A5-1 cells were synchronized and held in the S phase with the DNA-polymerase inhibitor aphidicolin. Microarray analysis and immunoblotting then were repeated. Under these conditions, we observed the attenuation of geminin RNA (Fig. 2A). Additionally, geminin protein levels also were attenuated (Fig. 2B). Therefore, the influence of RB upon geminin is not through indirect changes in cell cycle distribution.

Geminin Is Deregulated upon Loss of RB—Because we observed that geminin is repressed by RB, it would be expected that the loss of RB would lead to the deregulation of geminin expression. To carry out this analysis, we initially utilized MEFs harboring wild type or homozygously inactivated Rb. By microarray analysis, we observed a rather modest increase in geminin RNA levels in those cells deficient in RB (Fig. 3A, 1.6-fold change; p = 0.01). However, protein levels were significantly higher in the Rb-/- MEFs relative to their wild type counterparts (Fig. 3C). Recently, it has become clear that compensation can develop in cells lacking RB (18); therefore, we exploited a model to acutely knock out Rb. Murine adult fibroblasts were isolated from mice with Rb exon 3 flanked by loxP sites. In culture, Rb can be ablated efficiently from these cells through the use of cre-recombinase delivered by recombinant adenoviruses. When compared with cells infected with GFP-encoding adenovirus as a control, we observed that geminin RNA levels were induced in concert with the knock-out of Rb (Fig. 3B). Immunoblotting in this system also revealed elevated geminin protein levels following cre-infection (Fig. 3D). Thus, RB is required to maintain appropriate levels of geminin.

The Geminin Promoter Contains Several Conserved E2F Sites—As RB is known to function as a transcriptional repres-
sor, we sought to determine how geminin RNA levels are modulated by RB. RB is known to exert transcriptional repression via E2F DNA binding elements. Initially, we focused on the canonical promoter region upstream of the transcriptional start site (1, 2). Specifically, we cloned 520 base pairs upstream of the transcriptional start site (1, 2). This fragment contained a putative E2F site at −306 bp upstream of the trans-

Fig. 5. The first intron of geminin is critical for regulation by the RB pathway. Reporter constructs were generated by PCR amplification of mouse genomic DNA and cloning into the pGL2-Basic luciferase reporter vector. Exon 1 (clear boxes) is indicated along with the transcriptional start site (forward arrow), and the numbering of E2F sites (darkened boxes) indicates the distance from the transcriptional start site in base pairs. The beginning of coding sequence is indicated by the shaded box. A, reporter construct mGmnn (−521 to 1696) including the region cloned previously (Fig. 4) and the entire first intron. B, luciferase reporter activity for mGmnn (−521 to 1696). C, reporter constructs mGmnn (+1160 to 1696, +871 to 1696, and +181 to 1696) containing deletions of the first intron. Construct mGmnn ΔE2F contains a mutation in each of three E2F sites. D, reporter activity for these clones in the A5-1 cell line. E, rat1 cells were transfected with pGL2-mGmnn (+181 to 1696) and E2F2 expression plasmids. Activity is relative to the reporter without E2F2 co-transfection. Error bars represent the mean ± S.E. between samples. F, Rat1 cells were transfected with pGL2-mGmnn (+181 to 1696) and held in low serum for 96 h. Cells were returned to 10% serum for 0–18 h and harvested for reporter assays or fluorescence-activated cell sorter assays. Luciferase activity above the 0-h time point is shown in blue. The percentage of cells in the S phase calculated by flow cytometry is shown in red and represents 10^6 cells analyzed/time point.
The reporter was transfected into A5-1 cells that were then grown in the presence or absence of dox for 24 h. Repression by active RB was measured as the change in relative luciferase activity in the absence of doxycycline. Surprisingly, although this construct harbored significant luciferase activity, it was not repressed by active RB (Fig. 4B).

To extend our analysis for E2F-regulatory elements, we used the TraFaC program (trafac.chmcc.org) (19) to search for additional E2F motifs within the murine geminin genomic sequence. This analysis identified four E2F sites that were clustered around the first exon (Fig. 4, C and D). Many other putative binding sites for other transcription factors also were identified (data not shown), because the control of any given promoter is ultimately very complex. Because of our observations that RB is affecting geminin expression and because of the canonical role of E2F in RB-mediated repression, we have focused this analysis on E2F binding sites. TraFaC alignment of the genomic regions −1 kb upstream and downstream of the first exon in mouse, rat, and human showed that the E2F sites identified in mouse corresponded to similar sites in rat (Fig. 4C) and in human (data not shown). A compositional similarity between species for the presence of E2F sites further supports an important role for the RB/E2F pathway in the control of geminin expression. Additionally, this analysis suggested that the intragenic E2F sites downstream of the transcriptional start site may play a significant role in geminin control.

Active RB Represses Geminin through Intragenic E2F Sites—To address the role of all of the identified E2F sites, we cloned a larger region of geminin sequence and generated a reporter construct containing all four of the clustered E2F sites in mouse (Fig. 5A). In the reporter assays, the activity of this reporter was repressed strongly by active RB (Fig. 5B). These results suggested that the intragenic E2F sites are responsible for the observed repression. We then proceeded to generate deletions of this reporter to identify critical E2F sites. Initially, we focused on a specific intragenic E2F site just upstream of the coding region (exon 2) and found that in isolation it exhibited a relatively weak repression (Fig. 5, C and D). Similarly, the two downstream E2F sites in combination were only weakly repressed by PSM-RB. In contrast, the combination of all three E2F sites found in intron 1 resulted in strong repression equivalent to the repression observed with the larger geminin fragment. This reporter was activated strongly by the ectopic expression of E2F2, emphasizing the importance of the intragenic E2F sites in the control of geminin expression (Fig. 5E). These results suggest that the E2F sites downstream of
Repression of Geminin by RB

To verify that our geminin reporter with intact E2F sites is regulated by endogenous RB during the course of the cell cycle, transfections were carried out in Rat1 fibroblasts. These cells were held for 96 h in serum-deficient (0.01%) medium to synchronize the majority of cells in G1. Cells were stimulated with –10% serum for 0–18 h and harvested for flow cytometry and reporter assays. The strongest activation of the reporter occurred at 12 h after release from G1, just preceding the entry of most cells into S phase (Fig. 5F).

The Response of the Geminin Promoter to RB Is Dependent upon E2F—To investigate whether the response of the geminin promoter to RB is dependent on the E2F sites, we used site-directed mutagenesis to disrupt the three sites as shown in Fig. 6, A and B. Rat1 cells then were transfected with either pGL2-mGmnn (+181 to 1696), pGL2-mGmnn ΔE2F, PSM-RB, or PSM-RB and E2F2 expression plasmids (Fig. 6C). The wild type reporter was active and repressed by PSM-RB. This repression was alleviated mostly by cotransfection with E2F2, indicating that the observed repression occurs via E2F. The ΔE2F reporter was not significantly influenced by PSM-RB. These results demonstrate that the repression of the geminin promoter by active RB is mediated through these E2F sites.

The Geminin Promoter Is Occupied by E2F—To determine the ability of E2F proteins to occupy the geminin promoter, we used chromatin immunoprecipitation assay at the E2F sites in the rat promoter (Fig. 7A). The intragenic region of this promoter was repressed by RB in the A5-1 cell line similarly to the mouse promoter (Fig. 7B). The primers were designed to amplify pieces of the promoter containing the different E2F sites or to amplify a region −9 kb upstream of the transcriptional start site, which we determined by sequence comparison to be nonconserved and devoid of transcription factor binding sites (data not shown). As seen in Fig. 7C, E2F4 occupied predominantly E2F sites at +12, +324, and +708 of the rat geminin sequence. These results are consistent with the findings in mouse reporter assays confirming that the intragenic E2F sites are responsible for modulating transcription.

DISCUSSION

Here we show that geminin is regulated transcriptionally by the RB/E2F pathway. In the presence of a constitutively active RB allele, geminin RNA levels are repressed strongly regardless of cell cycle position. Similar repression is observed when endogenous RB is activated by p16INK4A overexpression. Conversely, in RB-deficient cells, geminin expression is derepressed. Therefore, geminin RNA and protein levels are regulated via RB. To delineate the mechanisms of regulation, we investigated E2F sites in the geminin promoter region. Typically, RB exerts its influence on gene expression through E2F sites relatively close to the transcriptional start site. Surprisingly, we failed to observe any repression with reporter constructs surrounding this conventional region of regulation. Cross-species analyses identified highly conserved E2F sites in proximity of the first exon of geminin, and reporter analyses confirmed that this region facilitated RB-mediated repression. Inactivation of endogenous RB at the G1/S transition resulted in the activation of a reporter construct, and the mutation of the E2F sites in the first intron prevented the repression of the reporter by PSM-RB. Additionally, chromatin immunoprecipitation confirms that E2F proteins are bound to these intragenic regions. These findings demonstrate that intragenic sequences play an important role in the regulation of geminin transcription.

The expression of geminin is controlled by the intricate interplay of transcriptional and proteolytic regulatory mechanisms. Degradation of geminin at the metaphase to anaphase transition has been shown to rely on the anaphase-promoting complex (1, 4). Therefore, the regulation of geminin at the transcriptional level by RB represents an additional control...
mechanism. RB is dephosphorylated and activated at the M/G1 transition when geminin levels decrease, and likewise, RB is phosphorylated and inactivated at the G1/S transition when the levels of geminin transcript increase and the protein begins to accumulate. We propose that RB functions in concert with the anaphase-promoting complex to maintain low levels of geminin throughout G1. The biological importance of low geminin levels through G1 has been indicated by several studies. McGarry and Kirschner (1) injected dividing Xenopus embryos with a stable mutant of Xenopus geminin lacking all 9 amino acids of the destruction box (geminin 
DEL). Although cell division continued, the resulting daughter cells were anucleate. Quinn et al. (23) identify the Drosophila geminin homolog and observe that overexpression causes the cells of Drosophila embryos to enter mitosis without replicating their DNA, resulting in a metaphase arrest and apoptosis. Numerous studies have shown that exogenous geminin blocks DNA licensing and replication (1, 5, 23–29). Furthermore, a recent study (30) shows that during the Xenopus embryonic cell cycle, 30–60% of endogenous geminin protein was not degraded at anaphase. The remaining geminin was altered in some way to prevent its degradation. The enhanced geminin levels observed in 
Rb
-lox/lox MEFs and 
Rb
-null MEFs and 
Rb
-deficient cells clearly exhibit defects in the critical licensing factor geminin. These analyses underscore the complexity of RB signaling both in mechanism of repression (through intragenic E2F sites) and diverse target spectrum (through the regulation of an inhibitor of DNA replication).

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