RB Inhibition of Cellular Proliferation during S-phase and Origin Density Determination through Alterations in Nucleotide Pools

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RB Inhibition of Cellular Proliferation during S-phase and Origin Density Determination through Alterations in Nucleotide Pools

Submitted in Partial Fulfillment of the Requirements for Graduation With Honors to the Department of Natural Sciences at Carroll College, Helena, Montana

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5 April 2004
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ABSTRACT

The retinoblastoma tumor suppressor protein, RB, is a negative regulator of the cell cycle that functions to prevent cells from entering S-phase when conditions are not appropriate for proliferation. It is thought that RB works to inhibit cellular proliferation in late G₁ phase of the cell cycle. Several recent discoveries indicate that RB may actually inhibit proliferation in S-phase rather than G₁. One of the biggest of these discoveries is that Cyclin E, an initiator of DNA replication, is produced in the presence of active RB. Cyclin E is responsible for the binding of cyclin dependent kinases at the end of G₁ and moving the cell into S-phase. At this point cyclin A, which is inhibited by active RB, takes over and continues the initiation of DNA replication. Because cyclin E is still produced in the presence of active RB, the cell cycle may not be inhibited until the initial stages of S-phase, where the lack of cyclin A prevents DNA replication.

A second function of RB is to prevent cellular proliferation not only by cyclin A inhibition, but also through the depletion of dNTP pools. When nucleotide levels decrease below a certain threshold, proliferation can no longer proceed. In conjunction with this idea, it was recently discovered that the number of utilized origins changes with increases or decreases in nucleotide levels, but the cell doubling time and length of S-phase remain the same.

Here I began preliminary testing on these two functions of active RB. First, RB actually inhibits cellular proliferation very early in the S-phase of the cell cycle, after primer formation but before processive elongation of replication forks. Second,
alterations made in nucleotide pools in the DHFR locus will decrease or increase origin utilization.
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INTRODUCTION

The cell cycle consists of four phases: \( G_1 \), \( S \), \( G_2 \), and mitosis. The cell is growing continuously during \( G_1 \), \( S \), and \( G_2 \), but DNA replication occurs only during S-phase. S-phase begins with \textit{initiation} where initiator proteins bind to double-stranded DNA helices and DNA helicase pries the strands apart by breaking hydrogen bonds between the bases. An RNA primer is then synthesized by RNA primase and later extended by DNA polymerase during \textit{elongation}.

During \( G_1 \), cells respond to stimuli that dictate whether the cell will proceed into S-phase or move out of the cycle and into a suspended quiescence (\( G_0 \)). The stimuli take the form of cytokines, and can function in the stimulation or prevention of proliferation (1). Several factors contribute to the functioning of the cyclins and cyclin dependent kinases (CDKs) to control cell division. One such factor is the retinoblastoma tumor suppressor protein (RB).

RB is a critical negative regulator of the cell cycle that functions to prevent cells from entering S-phase when conditions are not appropriate for proliferation (2-4). The protein also functions as a key player in the prevention of cancerous cell formation (1). Due to its function as a cell cycle inhibitor, RB is used extensively by research groups to experimentally control cell proliferation (2-4). Its use is especially important to medical researchers trying to find cures for cancer and other diseases like diabetes (5).

Recent analysis has shown that RB functions to inhibit proliferation in two ways (Figure 1). Angus et al. did extensive research in the inhibition of the cell cycle through
the repression of the E2F family of transcriptional regulators (2,4), and in inhibition that occurs through depletions of dNTP pools (3).

![Diagram](image)

**Figure 1: RB Inhibition of Proliferation.** RB functions to inhibit cellular proliferation via two different mechanisms – restriction of E2F targets like CDK2 and depletion of dNTP pools

1) **Repression of E2F:** The CDKs responsible for cellular passage into S-phase are regulated specifically by cyclins D, E, and A. Cyclin D binds CDK4 or CDK6, which are responsible for moving cells out of G0 and also for phosphorylating RB (1). Cyclin E is responsible for binding CDK2 and moving the cell into S-phase *initiation*, where the RNA primer is synthesized along the DNA template strand (6). Cyclin A then binds CDK2 and allows for PCNA (proliferating cell nuclear antigen) to attach at the beginning of *elongation*, which will allow the extension of the primer (6).

The E2Fs are a family of transcriptional regulators that bind to the promoters of genes that encode a variety of proteins required for S-phase entry including the cyclins and CDKs (6). In G0 and early G1, RB is hypophosphorylated and active when it binds to and forms a transcriptional repressor complex with an E2F protein, thereby preventing the synthesis of S-phase proteins (6). Once the cell is ready to enter S-phase, RB is phosphorylated and E2F is released so it can now bind to the promoter regions of essential S-phase proteins (2).
CyclinA-CDK2 is a cyclin-kinase complex regulated by E2F that functions in the activation of the sliding clamp protein PCNA (3). Extensive studies have shown that all replication downstream of PCNA is inhibited in the presence of active RB (3). Replication upstream of PCNA includes only those events of initiation, as PCNA is attached at the beginning of elongation after RNA primer formation (6). This may be an indication that RB does not inhibit cellular proliferation in G1, but instead at the very initial stage of S-phase after primer formation has occurred.

Cyclin A and cyclin E have long been thought to be blocked by active RB because they are both encoded by members of the E2F family of regulators (4). It has been recently shown that RB blocks cyclin A but does not block cyclin E (2). Sever-Chroneos et al. (2) and Feliers et al. (5) both found that cyclin E was still produced in the presence of activated RB, though neither would explain why. If cyclin E is produced in the presence of active RB, the cell should be able to move into the initiation period of S-phase, just prior to PCNA binding. This discovery is another indication that the cell cycle may not be inhibited until the elongation stage of S-phase, where the lack of cyclin A prevents DNA replication through PCNA binding.

RNA primase is needed at the start of replication to synthesize short RNA primers, which can then be elongated by DNA polymerase. The switch from unwinding DNA and synthesizing primers to the processive elongation of replication forks requires Cyclin A. Therefore, I hypothesize that RB allows initiation of replication but prevents this switch.

2) Depletion of dNTP pools: Angus et al. (3) recently found that active RB induces an imbalance in dNTP pools, concurrent with the inhibition of cellular
proliferation. Subtle changes in the levels of dNTPs can have a drastic effect on DNA replication. For example, as active RB worked to deplete the levels of dNTPs, proliferation came to a halt, as a threshold was reached and there were not enough nucleotides for replication to occur (2).

Based on these observations, Anglana et al. (7) observed the effects of increasing the levels of dNTPs in a given cell line. Initially, this lab group mapped out the locations of all the origins on a particular locus (AMPD2 – adenylate deaminase). Next, they lowered and raised the amounts of dNTPs and discovered that fork movement and progression rate are largely determined by the size of the nucleotide pools on the AMPD2 locus. Their results revealed that the average fork speed increased markedly in cells grown in the presence of adenine and uridine (increased dNTP pools), while the doubling time and S-phase duration remained unchanged. This means that the number of origins being used is increasing or decreasing, depending on the availability of nucleotides, in order to keep the length of S-phase constant. My second hypothesis is that the same will hold true for the DHFR (dihydrofolate reductase) locus, indicating that controlled origin utilization to ensure a constant S-phase time may in fact be a universal trend
MATERIALS AND METHODS

**HD-1 Cell Culture**: HD-1 (Hyg16-Rb delta cdk) cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (fetal bovine serum), 1% non-essential amino acids, 1% penicillin/streptomycin, 400μg/mL G418, 250μg/mL Hygromycin B, and 0.1 μg/mL Dox (doxycycline).

**Isoleucine Starvation**: HD-1 cells were starved for 45 hours in media lacking isoleucine (isoleucine− media: Earle's balanced salt solution, sodium carbonate, MEM vitamin mix, amino acid concentrate without isoleucine, FBS, 1% penicillin/streptomycin, Hyg B, G418, and Dox). Prior to starvation, 4x10^6 HD-1 cells were loaded onto coverslips in a 10cm dish with 10mL complete media (HD-1 cell culture media). This preparation was incubated in 5% CO₂ at 37°C overnight. Once the cells have reached a 90% confluence, starvation can begin. The complete media was removed and the cells were washed twice with isoleucine− media. Finally, 10 mL of isoleucine− media were added to the dish, which was incubated in 5% CO₂ at 37°C for 45 hrs.

**Release of mutated RB**: At the end of starvation, the cells were synchronized in G₁ so the isoleucine− media was removed and replaced with media used for cell culture minus the Dox. Coverslips were collected at -2, 0, 3, 5, 7, 11, and 15 hrs (time 0 represents the time at which the cells were removed from isoleucine− media). It was necessary to remove Dox from the media before starvation was completed (time 0) to allow for the desired effects from RB. Dox removal times used were 0, -2, and -4 hrs.
BrdU Incorporation: The coverslips were collected, stained, and then observed under a microscope for BrdU (bromodeoxyuridine) incorporation. It was important to keep the cells in the dark as much as possible as they are light sensitive. To stain, 1.5mL complete media and 4.5μL of 10mg/mL BrdU were instantly added to the freshly collected coverslips and incubated at 37°C for 30 minutes. The media was removed and the cells were washed twice with PBS (phosphate-buffered saline). They were then fixed with cold 70% ethanol and stored at 4°C until ready to finish the staining procedure. The ethanol was removed and the system was washed twice with PBS. HCl (1.5 M) was added and the system was incubated for 30 minutes at room temperature. The system was washed twice with PBS (3 minutes/wash). Anti-BrdU antibody in PBS-goat serum (1:10 dilution) was added at approximately 4μL/cover and incubated at room temperature for 1-2 hours in the dark. The slips were washed three times with PBS for 3 minutes/wash. Texas red-anti-mouse antibody in PBS-goat serum (1:100 dilution) was added at approximately 4μL/cover and incubate at room temperature for 1 hour in the dark. The slips were then washed three times with PBS for 3 minutes/wash. These coverslips were also stained with DAPI and then mounted on slides using vectashield at approximately 2μL/cover. Note: PBS-goat serum is a 1:10 dilution of serum stock and 10mg/mL DAPI is at a 1:3000 dilution in vectashield.

Cell Counts: Coverslips were counted for a percentage of cells showing an incorporation of BrdU. A total of 100+ cells were counted on each of two coverslips for each time point. Final percentages were averaged for a final value.

Serum Starvation: This procedure is similar to the isoleucine starvation procedure with the exception of the media used. Serum starvation media contains DMEM with
0.1% FBS, 1% non-essential amino acids, 400μg/mL G418, 250μg/mL Hyg B, and 0.1 μg/mL Dox. Coverslips were collected and stained in the same methods as previously described.

*Hydroxyurea Concentration:* CHO400 (Chinese hamster ovary) cells were grown in complete media (DMEM containing 10% FBS, 1% non-essential amino acids, and 1% penicillin/streptomycin) until they reached a 90% confluence. The cells were then split into new dishes at a 1:10 ratio and allowed to grow for 48 hours in the presence of various concentrations of hydroxyurea (0mM, 0.1mM, 0.3mM, 0.5mM, 1.0mM, or 1.5mM).
RESULTS AND DISCUSSION

*RB inhibition of cellular proliferation during S-phase:* HD-1 cells contain a gene that encodes for a mutant RB that cannot be phosphorylated. The RB remains in a fixed active state. In the presence of Dox, the promoter of this gene is blocked and the mutant RB cannot be synthesized. As soon as Dox is removed, the hypophosphorylated RB is encoded and synthesized.

The cells were starved in an isoleucine- media in order to synchronize them in $G_0$. If the cell does not have all of the available amino acids, it cannot proceed into S-phase and instead moves into the suspended state. The mutant RB was synthesized in the presence of complete media after the cells were synchronized and its effects were observed. A BrdU staining procedure was used to highlight the cells that were going through S-phase. BrdU labels the DNA in these cells and BrdU antibodies were used so the cells would be visible under a microscope.

Initially, I wanted to find a time to remove Dox during the isoleucine starvation that would give a consistent BrdU incorporation value less than 5% for all remaining time points. This would indicate that less than 5% of the cells were going through S-phase and that the mutant RB was working. Though I did manage to gain consistency in all time points by removing Dox four hours before starvation completion, I was unable to keep the value less than 5%. Figure 2 provides a visual representation of the data I collected over 10 weeks with Dox removal times of 0, -2, and -4 hours.
Figure 2: RB Inhibition of Cellular Proliferation. Cell counts at varying time points. Dox was removed at the completion of starvation (0 hours) as well as two and four hours prior to starvation completion. A best fit line was drawn through the data points.

The trend lines on the graph do indicate that the mutant RB is working to slow down cellular proliferation. A control was run in the constant presence of Dox to view what typical cellular proliferation rates would look like in the absence of the mutant RB. My results are similar to those found in other studies in regards to normal proliferating cells, in that the rate of proliferation begins to drastically increase around 7 hours (8). In the experimental trials, the rate of cell division has been greatly reduced by the mutant RB. Dox removal 4 hours prior to the completion of starvation provides the most linear trend in terms of proliferation, which was the desired effect. This trend indicates that the rate of cell division is not increasing at all, and that the mutant RB is working. Unfortunately, the rate of proliferation is well above 5%.

RB appears to have inhibited the rate change as the difference between the control and experimental cells becomes apparent after hour 7. Figure 3 is a visual representation of the control and experimental cells at hour 7.
Figure 3. BrdU incorporation at 7 hours. Images taken of cells fixed at the 7 hour time point from the control and Dox removal at -4 hours coverslips. The photographs indicate that both control and experimental cells are relatively even in terms of % of cells in S-phase.

A DAPI stain labels the nucleus of all cells present and BrdU only labels those cells going through S-phase. At this time point, both the control and experimental cells are dividing at a similar rate. After 7 hours, the effects of the mutant RB are made apparent. The largest difference in percentage of cells in S-phase between the control and experimental cells can be seen at 15 hours (Figure 4). At this time point, more of the control cells are entering S-phase than the experimental cells. The mutant RB has slowed down the rate of cell division in the experimental cells.
Due to the fact that the desired BrdU incorporation of less than 5% was not achieved in the RB cells, three options were considered to fix the problem. First, Dox may need to be removed from the media at a time point prior to -4 hours. Second, a different starvation technique may need to be utilized. Third, starvation time may need to be lengthened.

As a result of time constraints, I was only able to test option numbers one and two. Dox was removed from the isoleucine minus media 8 hours before starvation completion. There was no change in the data. HD-1 cells continued to incorporate BrdU at levels higher than 5%. I also made some initial steps for a 45 hour serum starvation involving 0.1% FBS. I was unable to tabulate the results of this experiment.

Overall, I was unable to fully determine if RB inhibits cellular proliferation in G₁ as previously assumed, or if it inhibits proliferation very early in S-phase, after primer formation but before processive elongation of replication forks. Once the initial problem
of gaining BrdU incorporation levels that are consistently below 5% for all 7 time points is worked out, experiments could be performed to determine if RNA primers have been assembled prior to RB inhibition. One such experiment would involve the addition of a nucleotide cocktail and DNA polymerase to the inhibited cells. If primers are present, DNA polymerase would promote elongation and DNA would be detectable on a gel, indicating that RB inhibition occurs during S-phase.

*Origin density determination through alterations in nucleotide pools:* Recent data indicates that an increase or decrease in nucleotide levels does not change the overall length of S-phase. This research points to the idea that as nucleotide levels increase, elongation occurs faster so less origins are utilized in order to keep S-phase at a constant length. The opposite is also true for decreased levels of nucleotides, in that more origins are utilized. The cell has some mechanism of controlling origin use in order to maintain the length of S-phase.

The locus used in the previous study was AMPD2. The purpose of my studies would be to repeat the same experiments on the DHFR locus. CHOC400 cells were utilized in my experiment as they have an amplified DHFR region.

The first step was to find the appropriate concentration of HU (hydroxyurea) that would decrease the number of nucleotides present in solution the most. HU functions to deplete the number of nucleotides in solution, which in turn slows the rate of cell division. Experimental cells were grown in complete medium with five different concentrations of HU. Growth rates were compared between the experimental cells and the control cells grown only in complete medium. Division seemed to have slowed down
the most in a 1mM concentration of HU (Table 1). This would be the concentration I would use for the rest of the experiments.

**Table 1. Cell growth in the presence of hydroxyurea.** Cell division slowed the most in a 1.0mM concentration of HU.

<table>
<thead>
<tr>
<th>HU Concentration</th>
<th>Vs. the Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>same confluency as the control</td>
</tr>
<tr>
<td>0.3 mM</td>
<td>slightly decreased confluency</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>decreased confluency</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>greatly decreased confluency (~10%)</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>same confluency as the control</td>
</tr>
</tbody>
</table>

CHO400 cells were then grown in the presence of adenine and uridine (50µM) or hydroxyurea (1.0mM) in accordance with the procedure used by the Debatisse lab (7). I was only able to make initial steps toward this hypothesis. I was able to freeze cells grown for 21 hours in 1mM HU as well as cells grown for 48 hours in 50µM adenine and 50µM uridine for future tabulations. The next step would be to permeate the cells with radioactively (³²P) labeled nucleotides and map out the origins using an in vivo ELFH assay.

If my hypothesis is correct, more origins should be utilized in the HU cells because there is a decrease in the nucleotide concentrations. Fewer origins should be utilized in the adenine/uridine-enriched cells when compared to normal CHO400 cells. These results, if seen, will indicate that the trend seen on the AMPD2 locus is also seen on the DHFR locus and may be universal for the entire genome.
A malfunctioning RB protein is a key component in many types of cancer including retinoblastomas, osteosarcomas, carcinoid tumors, and small-cell lung cancers (1). The future results of this experiment could prove that the textbooks are incorrect and influence upcoming experiments involving the RB protein. An eventual understanding of the mechanisms underlying RB function may change the way researchers are looking at cancer and get them one step closer to a cure.
REFERENCES


