The retinoblastoma tumor suppressor targets dNTP metabolism to regulate DNA replication

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RUNNING TITLE: RB-mediated depletion of dNTP pools
SUMMARY:

The retinoblastoma tumor suppressor, RB, is a negative regulator of the cell cycle that is inactivated in the majority of human tumors. Cell cycle inhibition elicited by RB has been attributed to the attenuation of CDK2 activity. Although ectopic cyclins partially overcome RB-mediated S-phase arrest at the replication fork, DNA replication remains inhibited and cells fail to progress to G2 phase. These data suggest that RB regulates an additional execution point in S-phase. We observed that constitutively active RB attenuates the expression of specific dNTP synthetic enzymes: dihydrofolate reductase (DHFR), ribonucleotide reductase (RNR) subunits R1/R2 and thymidylate synthase (TS). Activation of endogenous RB and related proteins by p16INK4a yielded similar effects on enzyme expression. Conversely, targeted disruption of RB resulted in increased metabolic protein levels (DHFR, TS, RNR-R2) and conferred resistance to the effect of TS or RNR inhibitors that diminish available dNTPs. Analysis of dNTP pools during RB-mediated cell cycle arrest revealed significant depletion, concurrent with the loss of TS and RNR protein. Importantly, the effect of active RB on cell cycle position and available dNTPs was comparable to that observed with specific antimetabolites. Together, these results show that RB-mediated transcriptional repression attenuates available dNTP pools to control S-phase progression. Thus, RB employs both canonical CDK/cyclin regulation and metabolic regulation as a means to limit proliferation, underscoring its potency in tumor suppression.
INTRODUCTION:

The retinoblastoma tumor suppressor (RB) functions as a negative regulator of cell cycle transitions (1-5). Due to its frequent inactivation in tumors (>60%), it is highly relevant to determine how RB functions to inhibit cellular proliferation and to elucidate its interaction with chemotherapeutic drugs.

Biochemically, RB functions as a transcriptional co-repressor that mediates the inhibition of cell cycle progression (1-5). RB interacts with multiple cellular proteins, including the E2F family of transcriptional regulators (6). In addition to binding E2F, RB also interacts with histone deacetylase (HDAC) and SWI/SNF chromatin remodeling proteins to establish a repressor complex on the promoters of E2F-regulated genes (3,7,8). This activity of RB is critical for cell cycle inhibition. In G0 and early G1, RB is hypophosphorylated and forms transcriptional repressor complexes to inhibit cell cycle progression. However, in response to mitogenic signaling, CDK/cyclin complexes phosphorylate RB (9). Phosphorylation disrupts the association of RB with its interacting proteins, thereby alleviating transcriptional repression of E2F-regulated genes and facilitating cell cycle progression (1-5).

Targets of E2F are known to encompass a variety of proteins involved in cell cycle progression (6,10,11). Consistent with the role of RB as a repressor of E2F, in disparate settings the expression/activity of cyclin E, cyclin A and CDK2 have been attenuated during RB-mediated arrest. Since these gene products are requisite for progression through S-phase, it is clear that these targets are important participants in RB-mediated cell cycle inhibition (2,12,13). Throughout S-phase, discrete origins of replication fire, and components of the DNA polymerase holoenzyme are sequentially
recruited to these sites (14). The binding of the sliding clamp protein PCNA to chromatin enables processive DNA synthesis and represents one of the final stages of this assembly (15). Consistent with the idea that RB regulates DNA replication, the expression of an active RB allele has been shown to specifically disrupt the association of PCNA with chromatin. Demonstrating the critical nature of CDK2 as a target of RB, PCNA activity was completely restored by the ectopic activation of CDK2 in the presence of active RB (16). Interestingly, although replication machinery was restored by CDK2 and some DNA synthesis occurred, replication was incomplete. These observations indicate that RB regulates S-phase through an additional mechanism independent of CDK2 activity.

Here, we define a CDK2-independent pathway through which RB regulates DNA replication, by controlling dNTP pools. We show that RB is required to maintain the relative expression of dNTP metabolic enzymes in proliferating cells, as loss of RB results in their deregulated expression and resistance to dNTP pool depletion. Conversely, activated RB completely attenuates enzyme expression, limiting available dNTP pools. The inhibitory effect of RB in this context is analogous to specific antimetabolite chemotherapeutics. Thus, RB impinges on DNA replication not only through canonical CDK/cyclin regulation, but also through the metabolic limitation of DNA precursor molecules.
MATERIALS AND METHODS:

Cell culture, adenoviral infections, and antimetabolite treatment.

Rat-16 and A2-4 cell lines were cultured and infected with cyclin E-encoding adenovirus (Gustavo Leone, Ohio State University) as previously described (16). Murine embryonic fibroblasts (MEFs) of either Rb<sup>+/+ </sup> or Rb<sup>−/−</sup> genotype, between passage 2 and 6, were obtained and cultured as described previously (17). CV1 cells were cultured as previously described (18). CV1 cells were infected with p16ink4a-encoding adenovirus (Timothy Kowalik, University of Massachusetts) as previously described (19). For flow cytometric analysis, dNTP pool analysis, and PCNA extraction of antimetabolite treated-cells, 10 µM methotrexate (MTX), 10 nM fluorodeoxyuridine (FdU), 10 µM 5-fluorouracil (5-FU), or 10 µM chlorodeoxyadenosine (CdA) was added to the cell culture medium for 16 hours. For BrdU incorporation, indicated concentrations of 5-FU or hydroxyurea (HU) were added to the culture medium for 16 hours. All compounds were purchased from Sigma.

Array analysis:

Total RNA was isolated from Rat-16 and A2-4 cells using Trizol (Gibco), according to the manufacturer’s protocol. RNA was then subjected to microarray analysis using Affymetrix genechips RatU34A, B, and C (Markey et al., submitted).

Immunofluorescence microscopy:

Approximately 10<sup>5</sup> A2-4 cells were grown on coverslips in six-well dishes. Approximately 10<sup>4</sup> MEFs were grown on coverslips in 24-well dishes. For bromodeoxyuridine (BrdU) incorporation, A2-4 cells or MEFs were pulse-labeled for 1 hour or 2 hours respectively, and stained as previously described (20). PCNA
immunofluorescence was performed on in situ extracted coverslips as previously described (16). PCNA antibody (PC10) was from Santa Cruz.

**Flow cytometry analysis:**

Subsequent to Dox removal, adenoviral infection, or antimetabolite treatment, cells were harvested by trypsinization, fixed with ethanol, stained with propidium iodide, and processed for flow cytometry as previously described (21).

**Immunoblotting and kinase assay:**

Immunoblotting was performed using standard procedures and the following antibodies from Santa Cruz: cyclin A (C-19), cyclin E (M-20 and HE12), CDK2 (M2-G), RNR-R1 (Y-16), RNR-R2 (I-15), and CDK4 (H-22). DHFR monoclonal antibody was from Transduction Laboratories. Polyclonal TS antibody was a kind gift from Masakazu Fukushima (Taiho Pharmaceutical) (22). PSM-RB and endogenous RB were detected using 851 antibody.

For in vitro kinase assays, cells were lysed and CDK2 immune-complexes were recovered and used to phosphorylate histone H1 substrate as previously described (20).

**dNTP pool extraction and analysis:**

Asynchronously proliferating Rat-16 or A2-4 cells were cultured as described. For each analysis, the cells in three 10-cm plates were washed with 5 ml each of cold PBS. Extraction was carried out with 3 ml per plate of ice-cold 60% methanol/1% toluene. Plates were incubated at -20°C for 2 hours, following which the fluid was recovered and each plate was washed with an additional 1 ml of the methanol/toluene solution. Following this, all suspensions and washes were pooled. The remainder of the extraction was carried out as described by Sargent and Mathews (23). Analysis of the
dNTP pools in each extract was carried out as described by Sherman and Fyfe (24). Reaction mixtures (50 µl) contained 100 mM HEPES buffer, pH 7.5, 10 mM MgCl₂, 0.1 U *E. coli* DNA polymerase I Klenow fragment (U.S. Biochemical), 0.25 µM oligonucleotide template, and 1 µCi [³H]dATP (Amersham) or dTTP (Moravek). Incubation was carried out for 60 minutes at 37°C.
RESULTS:

Cyclin E fails to overcome RB-mediated cell cycle inhibition:

It is well established that RB regulates S-phase by influencing CDK2 activity, as the ectopic expression of cyclins can promote S-phase entry in the presence of constitutively active alleles of RB (21,25,26). However, the replication is incomplete and cells fail to progress into G2 phase. Here, we sought to uncover the additional, CDK2-independent mechanism through which RB inhibits DNA replication. We utilized an established system in which the expression of a constitutively active allele of RB, PSM-RB, can block DNA replication (21). To verify these results and determine the action of PSM-RB on the biochemical activities associated with DNA replication, a cell line that inducibly expresses PSM-RB was utilized (16). In this rat fibroblast cell line (A2-4), PSM-RB expression was induced by removal of doxycycline (Dox) from the medium for 16 hours (Fig. 1A, compare lanes 1 and 2). Expression of PSM-RB led to an accumulation of cells with a 2N DNA content as determined by flow cytometry (Fig. 1C, mock-infected, compare +/-Dox).

To investigate the effect of cyclin E overproduction on this inhibition of cell cycle progression, a recombinant adenovirus encoding human cyclin E was employed. Ectopic expression of cyclin E was confirmed in infected cells by immunoblotting with an antibody specific for human cyclin E (Fig. 1A, lanes 3 and 4). To confirm that ectopic cyclin E expression restored CDK2 activity, we performed in vitro kinase assays (Fig. 1B). As previously observed, the expression of PSM-RB inhibited CDK2 activity (Fig. 1B, mock-infected, compare lanes 2 and 3). In contrast, overproduction of cyclin E in the presence of PSM-RB restored the in vitro activity of CDK2 complexes (Fig. 1B,
cyclin E-infected, compare lanes 5 and 6). While the adenoviral infection had no effect on the expression of PSM-RB (Fig. 1A, compare lanes 2 and 4), ectopic cyclin E expression partially overcame the PSM-RB-mediated arrest, as cells accumulated with greater than 2N DNA content (Fig. 1C, compare mock- and cyclin E-infected, -Dox). However, the cells co-expressing cyclin E and PSM-RB remained inhibited for DNA replication, failing to attain 4N DNA content. This was not merely a consequence of cyclin E overproduction, as cells expressing cyclin E in the presence of doxycycline exhibited normal cell cycle distribution (Fig. 1C, compare mock- and cyclin E-infected, +Dox). Consistent with inefficient S-phase progression, ectopic cyclin E expression in the presence of PSM-RB resulted in a punctate BrdU labeling pattern (Fig. 1D, compare mock- versus cyclin E-infected BrdU immunofluorescence) (21).

Expression of active RB specifically disrupts the activity of PCNA, the sliding clamp protein required for processive DNA synthesis (15,16). To determine whether the incomplete DNA replication observed upon ectopic cyclin E expression was due to a failure to restore PCNA activity, in situ extraction of asynchronously proliferating cells in the presence or absence of Dox was performed (Fig. 1E). This procedure removes soluble proteins, including inactive replication factors that are not associated with chromatin (27). As expected, the percentage of nuclei with detectable, chromatin-bound PCNA was reduced by PSM-RB expression (Fig. 1E, mock-infected, upper and lower panels, compare +/-Dox). In contrast, cyclin E efficiently restored PCNA binding in the presence of active RB (Fig. 1E, cyclin E-infected, upper and lower panels, -Dox). Therefore, although entry into S-phase was stimulated, PSM-RB-mediated inhibition of replication persisted in the face of cyclin E overproduction and tethered PCNA. These
data indicate that RB must regulate DNA replication at a second execution point, independent of CDK2 activity and downstream of PCNA loading.

**PSM-RB inhibits the expression and activity of dNTP metabolic enzymes:**

To identify this point, microarray analyses were utilized and data mined for enzymes that affect post-PCNA DNA replication (*e.g.* strand elongation). Asynchronously proliferating A2-4 cells readily accumulated with a 2N DNA content following removal of Dox from medium for 16 hours, while the parental cell line, Rat-16, was largely unaffected (Fig. 2A, compare A2-4 and Rat-16, +Dox and –Dox 16 hours). In fact, the Rat-16 cells readily proliferate in the absence of doxycycline (not shown), while the inhibition of cell cycle progression mediated by PSM-RB persisted throughout the course of our studies (Fig. 2A, compare A2-4, +Dox and –Dox).

Rat-16 and A2-4 cells were cultured in the presence or absence of doxycycline for 24 hours and total RNA was utilized to define targets that were reproducibly downregulated by the expression of PSM-RB using Affymetrix microarrays. Initially, the relative expression of candidate target genes was examined at the mRNA level as part of a genome-wide analysis of RB-mediated transcriptional repression. Strikingly, the relative mRNA levels of multiple enzymes involved in dNTP metabolism were repressed upon the expression of PSM-RB (Fig. 2B). As shown in Fig. 2B, removal of Dox from the medium had little effect on mRNA levels in Rat-16 cells, while the expression of PSM-RB in A2-4 cells led to the marked attenuation of dihydrofolate reductase (DHFR), thymidylate synthase (TS), and ribonucleotide reductase subunits R1 (RNR-R1) and R2 (RNR-R2). DHFR and TS are involved in the production of thymidine (28,29), while
RNR regulates the rate-limiting conversion of all ribonucleotides (NDPs) to deoxyribonucleotides (dNDPs) (30-33).

To determine whether these changes in mRNA levels led to significant changes in protein expression, relative enzyme levels in Rat-16 and A2-4 cells were determined by immunoblotting. For DHFR, we observed no change at the protein level coincident with cell cycle inhibition at 16 hours following removal of Dox from the medium (Fig. 2C, DHFR, compare A2-4 and Rat-16). However, at 72 hours after Dox removal, DHFR levels diminished in the presence of PSM-RB (Fig. 2C, compare A2-4, 16 to 72 hours - Dox). In contrast to DHFR, the expression of TS protein was rapidly attenuated by PSM-RB induction (Fig. 2C, compare A2-4 and Rat-16). Additionally, expression of both RNR subunits was strongly suppressed in the presence of active RB (Fig. 2C, compare A2-4 and Rat-16). Thus, the effect of RB on metabolic enzyme mRNA levels was consistent with the robust attenuation of TS and RNR protein.

To confirm that dephosphorylation of endogenous RB and related proteins could downregulate dNTP enzyme expression, we analyzed the effects of ectopic p16ink4a expression. P16ink4a inhibits CDK activity to prevent the phosphorylation of RB, p107, and p130, an event required for cell cycle inhibition (34,35). After a 16-hour infection, the expression of p16ink4a in CV1 cells was readily detectable by immunoblotting (Fig. 2D) and cell cycle inhibition was observed (data not shown). The expression of p16ink4a led to the dephosphorylation/activation of endogenous RB, as indicated by the accumulation of its hypophosphorylated form (Fig. 2D). Importantly, the protein levels of identified RB-repressed targets, RNR-R2, TS, and DHFR were significantly reduced
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These data indicate that the endogenous RB-family proteins target dNTP enzyme expression during cell cycle arrest.

If the diminution of TS and RNR proteins in A2-4 cells was responsible for the inefficient DNA replication in the presence of cyclin E, their levels should be unaffected by cyclin E. Indeed, analysis of RNR-R2 and TS revealed that protein expression remained attenuated by PSM-RB in the presence of ectopic cyclin E expression (Fig. 2E). Thus, cyclin E restores PCNA activity but fails to recover the expression of dNTP metabolic enzymes in the presence of active RB.

Loss of RB leads to deregulated dNTP enzyme expression and antimetabolite resistance:

To directly assess the action of endogenous RB on dNTP metabolic enzymes, murine embryonic fibroblasts (MEFs) of Rb+/+ or Rb−/− genotype were utilized (17). The loss of functional RB in MEFs has been shown to result in the overexpression of specific RB/E2F-regulated genes (36-38). As a positive control for deregulated expression, we analyzed the cyclin E protein levels in the MEFs (Fig. 3A, compare lanes 1 and 2) (37). In contrast, the levels of CDK4 were not deregulated. Analysis of the DHFR and TS protein levels by immunoblotting revealed that they were significantly increased in Rb−/− MEFs (Fig. 3A, compare lanes 1 and 2). Additionally, we observed increased abundance of the RNR-R2 subunit in Rb−/− MEFs (Fig. 3A, compare lanes 1 and 2). To determine the significance of this augmented enzyme expression in Rb−/− cells, we utilized the RNR inhibitor, hydroxyurea (HU) and the thymidylate synthase inhibitor, 5-fluorouracil (5-FU). Rb+/+ or Rb−/− MEFs were cultured in the presence of each antimetabolite and evaluated for their ability to incorporate BrdU. The inhibition of BrdU incorporation in
wild-type MEFs was dose-responsive to both 5-FU and HU treatment (Fig. 3B, upper and lower panels, black bars). In contrast, \( Rb^{-/-} \) MEFs continued to incorporate BrdU in the presence of either drug at the highest concentration utilized (Fig. 3B, upper and lower panels, gray bars). Thus, the action of 5-FU and HU is dependent on the metabolic enzyme levels imposed by RB.

**dNTP pool alterations during RB-mediated arrest:**

The data presented suggest that RB may regulate dNTP pools through modulation of TS and RNR expression. To test this hypothesis directly, cellular dNTP pools were analyzed in the presence or absence of active RB (23). The control cell line, Rat-16, did not significantly alter the absolute or relative levels of dNTP pools upon Dox removal (Fig. 4A and B, Rat-16). In contrast, a substantial decline in absolute dNTP levels and a significant change in the relative pool composition were observed 16 hours after the removal of Dox in A2-4 cells (Fig. 4A and B, A2-4). In extracts from these RB-arrested cells, dATP levels were reduced 77%, dTTP levels were reduced 62%, and dGTP levels were reduced by approximately 50%. In contrast, the levels of dCTP did not appreciably change upon PSM-RB expression (Fig. 4A). This initial attenuation of dNTPs persisted throughout the RB-induced cell cycle arrest (Fig. 4A and B, A2-4). Thus, changes in the expression of metabolic enzymes by PSM-RB correlated with the significant reduction in specific dNTP levels.
Action of RB is comparable to those of antimetabolites that inhibit DNA replication:

To assess the biological relevance of the RB-mediated dNTP pool depletion, several common antimetabolic drugs were employed that specifically inhibit replication by attenuating dNTP pools. For example, methotrexate (MTX) inhibits DHFR activity while fluorodeoxyuridine (FdU) and 5-FU target the activity of thymidylate synthase (29,39). In addition, chlorodeoxyadenosine (CdA) is thought to trigger cell cycle inhibition primarily by blocking RNR function (40). As such, these compounds halt DNA replication by limiting dNTPs via specific enzyme targeting. A2-4 cells were cultured in the presence of doxycycline (PSM-RB expression off) and treated for 16 hours using the indicated concentrations of each antimetabolite. Following treatment, cells accumulated with G1/S DNA content, indicative of a DNA replication block (Fig. 5A). To assess the effect of antimetabolite treatment on the replication machinery, PCNA activity was examined. In the presence of MTX and CdA, PCNA was efficiently tethered (Fig. 5B, compare +/- Dox to MTX and CdA). These observations are analogous to those seen upon ectopic cyclin E expression in RB-arrested cells (Fig. 1E).

As has been described, these antimetabolites ultimately exert their effect by limiting available DNA precursors. As shown in Fig. 5C, MTX, FdU, and 5-FU efficiently attenuated dTTP levels (88%, 86%, and 68% respectively) when compared to the untreated control. In comparison, CdA treatment only attenuated the levels of dATP (68%) (Fig. 5C). Thus, changes in dNTP levels of this magnitude were sufficient to inhibit DNA replication (Fig. 5A). Importantly, the effects of these antimetabolites on dNTP levels were comparable to those observed in response to PSM-RB expression (Fig.
5C, compare antimetabolites to –Dox). These data indicate that the RB-mediated depletion of dNTP pools contributes to the inhibition of DNA replication.
DISCUSSION:

RB-mediated cell cycle inhibition occurs in response to anti-mitogenic signals, DNA damage, and other cellular stresses (1-5). The cell cycle arrest invoked by RB is thought to occur through the inhibition of CDK2 activity or the modulation of cell cycle regulatory factors (2,12,13). However, RB-mediated arrest can only be partially subverted by the ectopic expression of the CDK2 activators, cyclin E and cyclin A (16,21,25,26). Cyclin overproduction in the presence of active RB restores CDK2 activity and triggers S-phase entry; however, efficient DNA replication is not achieved. Analysis of the replication machinery indicated that PCNA tethering was restored, suggesting that downstream effects on the supply of dNTPs may be limiting. Here, we report that the expression of active RB downregulates the levels of both RNR subunits and TS. Targeted disruption of RB resulted in deregulation of TS and RNR-R2 protein levels and resistance to antimetabolites that target their enzyme activity. Active RB induced an imbalance of intracellular dNTP pools, concomitant with the inhibition of DNA replication. The effects of RB on cell cycle and dNTP levels were comparable to effects of antimetabolites that target RNR and TS activity. Thus, the RB tumor suppressor pathway regulates DNA replication via CDK2 modulation and the metabolic control of dNTP pools.

The function of RB to negatively regulate cellular proliferation is attributed to its transcriptional repression of E2F target genes (3). These E2F targets encompass a wide variety of cell cycle regulatory and metabolic enzymes (6,10,11). It has been viewed that the downregulation of cell cycle regulatory machinery is the primary means by which RB limits cell proliferation. Consistent with this, RB has been shown to inhibit
the expression of cyclin E, cyclin A, or CDK2 to impede S-phase progression (8,16,21,25,41-43). This has been demonstrated through the reduction in amount of target proteins and subsequent attenuation of CDK2-associated kinase activity. Since CDK2 activity is required for DNA synthesis, this represents a mechanism through which RB inhibits cell cycle progression (2,12,13,15). Consistent with this idea, ectopic expression of cyclins E or A can partially overcome the inhibition of DNA replication mediated by active RB alleles (16,21,25,26). However, replication is incomplete; cells accumulate with S-phase DNA content and punctate BrdU labeling is observed. Investigation of DNA replication machinery under these conditions indicated that PCNA is still associated with chromatin. PCNA is a component of the processive DNA polymerase holoenzyme and is one of the last regulatory effectors of DNA replication (15,16). Thus, the sustained inhibition achieved by PSM-RB in the presence of cyclin E represents a very late step in DNA replication and suggests that a specific action of RB may be to act downstream of the replication machinery to inhibit DNA synthesis. One of the few previously identified mechanisms through which replication is inhibited with PCNA tethered to chromatin is through the depletion of dNTP pools through the use of HU (44).

The relative levels of dNTPs and the regulation of their synthesis play a critical role in DNA replication (30,32,45). As such, expression of dNTP synthetic enzymes is cell cycle-regulated, with enhanced expression in S-phase. Even subtle changes in the levels of dNTPs can have a dramatic effect on DNA replication (45). For example, inhibition of RNR activity by 50% using CdA leads to marked inhibition of cell cycle progression (40). Additionally, dNTP levels vary within S-phase of the cell cycle (46);
these variations may be responsible for changes in the rate of DNA replication during S-phase ((47) and S.A. Martomo and C.K. Mathews, manuscript in preparation).

Consistent with the idea that the attenuation of dNTP metabolism could be a mechanism through which RB inhibits DNA replication, E2F can modify the transcription of several metabolic enzymes (10,11,48,49). Specifically, it has been demonstrated that ectopic expression of E2F can stimulate the expression of DHFR, RNR-R1, RNR-R2, TS, and thymidine kinase (TK) in quiescent cells (10). In fact, recent chromatin immunoprecipitation analyses have detected RB on the DHFR promoter at the G1/S transition (50). Thus, E2F activity is believed to maintain the relative levels of enzyme mRNA during cell cycle progression. Here, we evaluated whether RB could specifically attenuate the expression of metabolic targets as part of a program to inhibit DNA replication. We find that RB reduces the mRNA levels of dNTP synthetic enzymes, with RNR-R2 being the most strongly repressed and DHFR being weakly repressed. We show that active RB targets the protein levels of RNR-R1, RNR-R2, DHFR and TS to effectively limit their abundance. As may be expected for metabolic enzymes, the kinetics of DHFR attenuation were slow and did not correlate with cell cycle inhibition achieved by active RB. However, the RNR-R2, RNR-R1 and TS enzymes were significantly attenuated, concurrent with cell cycle inhibition. In addition, activation of endogenous pocket proteins by ectopic p16ink4a expression led to the loss of RNR-R2, TS, and DHFR. Thus, the depletion of metabolic enzymes mediated by active RB could participate in the inhibition of DNA replication by virtue of altered dNTP pools.
In keeping with the significant role of dNTP metabolism in replication control, a number of therapeutic drugs are utilized that target dNTP synthetic enzymes (28,29,39). These antimetabolites generally function as pseudo-substrates that poison their specific target enzymes, leading to the depletion of dNTPs and subsequent inhibition of DNA replication. One mechanism through which resistance to antimetabolites is achieved is through overexpression of the target enzymes. We found that Rb<sup>−/−</sup> MEFs significantly overproduced RNR-R2, TS and DHFR protein. Our data is consistent with prior studies demonstrating that loss of RB leads to deregulation of metabolic enzyme mRNA (38,51). Specifically, Almasan et al. showed that mRNA levels of both TS and DHFR were elevated in asynchronously proliferating Rb<sup>−/−</sup> MEFs compared to wild-type MEFs (38). We observed that Rb<sup>−/−</sup> cells were resistant to increasing doses of the TS inhibitor 5-FU that are known to block DNA synthesis. Furthermore, the increase in RNR-R2 seen in the absence of RB resulted in resistance to the specific RNR inhibitor, HU. These results complement prior studies demonstrating the resistance of RB-deficient cells to MTX and FdU (38,51). Thus, RB regulates the relative expression levels of a coordinate set of dNTP synthetic enzymes, thereby rendering cells resistant to a variety of antimetabolites.

Lastly, to directly assess the effect of RB on replication precursors, we analyzed dNTP pools. Surprisingly, no prior study has implicated a mammalian signal transduction cascade involved in cell cycle control to the level of dNTP and inhibition of DNA replication. In S. cerevisiae, several studies have demonstrated the involvement of SML1, an inhibitor of RNR, in the replicative response to DNA damage (52,53). As would be expected from the dramatic effects on protein expression, we find that dNTP pools are significantly reduced through the action of RB. The changes mediated by RB
are comparable in magnitude to the changes elicited by antimetabolites that inhibit key enzymes involved in dNTP metabolism. Importantly, the inhibition of replication observed by the use of these antimetabolites was accompanied by the retention of PCNA on chromatin. Thus, cells arrested by antimetabolites behave in a manner analogous to those inhibited for DNA replication with both PSM-RB and cyclin E.

In summary, our findings reveal dual roles for RB in DNA replication control: concurrent regulation of CDK2 activity and metabolic enzyme activity through transcriptional regulation.

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REFERENCES

FIGURE LEGENDS

Fig. 1.  Ectopic cyclin E promotes S-phase entry but fails to overcome RB-mediated cell cycle inhibition (A) Asynchronously proliferating A2-4 cells were either mock-infected (lanes 1 and 2) or infected with cyclin E-producing adenovirus (lanes 3 and 4) and subsequently cultured in medium with (lanes 1 and 3) or without (lanes 2 and 4) Dox for 16 hours. Equal amounts of total protein were resolved by SDS-PAGE, and PSM-RB, adenovirally-produced human cyclin E, or CDK4 respectively were detected by immunoblotting. (B) A2-4 cells were mock-infected (lanes 1-3) or infected with cyclin E (lanes 4-6) and subsequently cultured in medium with (lanes 1, 2, 4, and 5) or without (lanes 3 and 6) Dox for 16 hours. E1A (control, lanes 1 and 4) or CDK2 (lanes 2, 3, 5, and 6) was immunoprecipitated, and resultant immune-complexes were used in in vitro kinase reactions against histone H1. Reactions were resolved by SDS-PAGE and transferred to PVDF membrane. Phosphorylated histone H1 was detected by autoradiography. (C) A2-4 cells were infected and cultured as in (A) and then harvested by trypsinization, fixed, stained with propidium iodide, and analyzed by flow cytometry. Representative histograms are shown of 10,000 gated events. (D) A2-4 cells seeded onto coverslips were infected and cultured on as described in (A). Cells were pulse-labeled with BrdU for one hour, fixed, and stained for BrdU incorporation. Representative photomicrographs were taken at 63X magnification. (E) A2-4 cells grown on coverslips were infected and cultured as in (A). Coverslips were extracted, fixed with methanol, and processed for immunofluorescence using PCNA antibody. Values shown are the averages from at least two independent experiments with at least 200 cells counted per experiment. Representative photomicrographs were taken at 63X magnification.
**Fig. 2.** Active RB mediates repression of dNTP metabolic enzymes during cell cycle arrest. (A) Asynchronously proliferating Rat-16 (upper panels) or A2-4 (lower panels) were cultured in medium in the presence or absence of Dox. At the indicated time points, cells were harvested and an aliquot was fixed with ethanol, stained with propidium iodide, and processed for flow cytometric analysis. Representative histograms are shown from 10,000 gated events. (B) Asynchronously proliferating Rat-16 or A2-4 cells were grown either in the presence or absence of Dox for 24 hours. RNA was isolated, and utilized for microarray analysis on an Affymetrix gene chip. Shown for each gene is the fold-repression (average ± standard error) upon removal of Dox. Values shown are from two independent experiments. (C) Rat-16 (lanes 1-5) or A2-4 (lanes 6-10) cells from (A) were lysed, and equal amounts of total protein from each time point were resolved by SDS-PAGE. PSM-RB, DHFR, TS, RNR-R1, RNR-R2, and CDK4 were detected by immunoblotting. (D) Asynchronously proliferating CV1 cells were either mock-infected (lane 1) or infected with a recombinant adenovirus encoding p16ink4a (lane 2) and cultured for 16 hours. Whole-cell lysates were prepared and resolved by SDS-PAGE. P16ink4a, RB, RNR-R2, TS, DHFR, and CDK4 were detected by immunoblotting. (E) Equal amounts of protein from A2-4 whole-cell lysates from Fig. 1A were resolved by SDS-PAGE and RNR-R2 and TS were detected by immunoblotting.

**Fig. 3.** RB is required for DNA replication inhibition by antimetabolites. (A) Asynchronously proliferating wild-type (Rb+/+) or Rb-/- MEFs were harvested and equal
amounts of total protein were separated by SDS-PAGE. Cyclin E, DHFR, TS, RNR-R2, and CDK4 were detected by immunoblotting. (B) \( Rb^{+/+} \) and \( Rb^{-/-} \) MEFs were seeded at equal density onto coverslips in a 24-well dish. 5-FU (upper panel) or HU (lower panel) was added to the tissue culture medium at the indicated concentration for 16 hours. Cells were pulse-labeled with BrdU for 2 hours, fixed with formaldehyde, and processed for BrdU immunofluorescence. Values shown represent at least 200 cells counted.

**Fig. 4.** dNTP pool depletion during RB-mediated cell cycle arrest. (A) Asynchronous Rat-16 and A2-4 cells were either grown in the presence or absence of Dox for the indicated times. Samples were matched to those utilized for Fig. 2. Plates were washed twice with PBS and then extracted with ice-cold 60% methanol/1% toluene solution at -20°C for 2 hrs. Soluble extracts were lyophilized and the values for each dNTP were determined. Values shown are from at least two independent experiments. (B) Data collected from (A) was utilized to determine the total amounts of each dNTP from each time point. Percent contribution of each dNTP to the total pool is represented.

**Fig. 5.** Antimetabolites that target dNTP production mimic RB-mediated cell cycle arrest. (A) Asynchronously proliferating A2-4 cells were cultured in the presence of Dox alone (untreated) or either 10 \( \mu \)M methotrexate (MTX), 10 nM fluorodeoxyuridine (FdU), 10 \( \mu \)M 5-fluorouracil (5-FU), or 10 \( \mu \)M chlorodeoxyadenosine (CdA), each added for 16 hours. Cells were harvested and an aliquot was fixed with ethanol, stained with propidium iodide, and processed for flow cytometric analysis. Representative histograms are shown from 10,000 gated events. (B) A2-4 cells were seeded onto
coverslips and cultured in the presence (+Dox) or absence of Dox (-Dox) or in the presence of Dox and either chlorodeoxyadenosine (CdA) or methotrexate (MTX) for 16 hours. Cells were subjected to in situ extraction, fixed in methanol, and processed for immunofluorescent detection of PCNA. At least 150 cells were counted for each experiment. (C) At the indicated times, plates cultured as in (A) were washed twice with PBS and then extracted with ice-cold 60% methanol/1% toluene solution at -20°C for 2 hrs. Soluble extracts were lyophilized and the values for each dNTP were determined. Values shown are from at least two independent experiments. (D) Data collected from (C) was utilized to determine the total amounts of each dNTP from each time point. Percent contribution of each dNTP to the total pool is represented. (E) Inhibition of DNA synthesis by active RB has been shown to depend partially on the attenuation of CDK2 activity. This signaling pathway has been shown to disrupt the chromatin-binding activity of PCNA, the sliding clamp required for processive DNA synthesis. Data presented here demonstrate that RB can also regulate the levels of crucial dNTP synthetic enzymes such as RNR and TS. This signaling is likely responsible for the perturbation of dNTP pools seen upon the expression of active RB. The repression of dNTP pools is apparently independent of CDK2 activity, demonstrating two important pathways of RB-mediated DNA replication control.
Fig. 1

A

<table>
<thead>
<tr>
<th></th>
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<th>Dox</th>
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- PSM-RB
- Human cyclin E
- CDK4

B

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<tr>
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<tbody>
<tr>
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- Histone H1

C

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<td>- Dox</td>
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- # Cells

D

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<tr>
<td></td>
<td>Hoechst</td>
<td>BrdU</td>
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<tr>
<td>Cyclin E-infected</td>
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</table>

E

- % PCNA positive
- Tethered PCNA

- Dox: + - -
- Infection: Mock Cyclin E

Hoechst PCNA (tethered)
**Fig. 2**

**A**

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<tr>
<td>A2-4</td>
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<tr>
<td></td>
<td>16</td>
<td>24, 48, 72</td>
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**B**

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<tr>
<td>DHFR</td>
<td>1.14 ± 0.36</td>
<td>1.81 ± 0.17</td>
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<td>TS</td>
<td>1.10 ± 0.24</td>
<td>2.17 ± 0.03</td>
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<tr>
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<td>2.24 ± 0.26</td>
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<td>RNR-R2</td>
<td>1.21 ± 0.01</td>
<td>4.02 ± 0.17</td>
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<tr>
<td>GAPDH</td>
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**D**

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**E**

A2-4: Cyclin E-infected

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Fig. 2
Fig. 3
**A**

![Graph showing the amount of nucleotides (dATP, dTTP, dCTP, dGTP) over time with and without Dox.](image)

<table>
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<tr>
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**B**

![Pie charts showing nucleotide distribution in Rat-16 and A2-4 cells with and without Dox.](image)

- **Rat-16**
  - +Dox: 41%, dATP; 24%, dTTP; 27%, dCTP; 8%, dGTP
  - -Dox: 43%, dATP; 23%, dTTP; 25%, dCTP; 9%, dGTP

- **A2-4**
  - +Dox: 31%, dATP; 24%, dTTP; 30%, dCTP; 7%, dGTP
  - -Dox: 38%, dATP; 27%, dTTP; 57%, dCTP; 8%, dGTP

**Fig. 4**
Fig. 5
The retinoblastoma tumor suppressor targets dNTP metabolism to regulate DNA replication
Steven P. Angus, Linda J. Wheeler, Sejal A. Ranmal, Xiaoping Zhang, Michael P. Markey, Christopher K. Mathews and Erik S. Knudsen

*J. Biol. Chem.* *published online September 6, 2002*

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