Ribonucleotide Reductase Activity and Deoxyribonucleoside Triphosphate Metabolism during the Cell Cycle of S49 Wild-type and Mutant Mouse T-lymphoma Cells*

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We investigated deoxyribonucleoside triphosphate metabolism in S49 mouse T-lymphoma cells synchronized in different phases of the cell cycle. S49 wild-type cultures enriched for G1 phase cells by exposure to dibutryl cyclic AMP (Bt2cAMP) for 24 h had lower dCTP and dTTP pools but equivalent or increased pools of dATP and dGTP when compared with exponentially growing wild-type cells. Release from Bt2cAMP arrest resulted in a maximum enrichment of S phase occurring 24 h after removal of the Bt2cAMP and was accompanied by an increase in dCTP and dTTP levels that persisted in colcemid-treated (G2/M phase enriched) cultures. Ribonucleotide reductase activity in permeabilized cells was low in G1 arrested cells, increased in S phase enriched cultures and further increased in G2/M enriched cultures.

In cell lines heterozygous for mutations in the allosteric binding sites on the M1 subunit of ribonucleotide reductase, the deoxyribonucleotide pools in S phase enriched cultures were larger than in wild-type S49 cells, suggesting that feedback inhibition of ribonucleotide reductase is an important mechanism limiting the size of deoxyribonucleoside triphosphate pools.

The M1 and M2 subunits of ribonucleotide reductase from wild-type S49 cells were identified on two-dimensional polyacrylamide gels, but showed no significant change in intensity during the cell cycle. These data are consistent with allosteric inhibition of ribonucleotide reductase during the G1 phase of the cycle and release of this inhibition during S phase. They suggest that the increase in ribonucleotide reductase activity observed in permeabilized S phase-enriched cultures may not be the result of increased synthesis of either the M1 or M2 subunit of the enzyme.

The enzyme ribonucleoside diphosphate reductase (EC 1.17.4.1) catalyzes reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are then converted to the precursors for DNA synthesis. Ribonucleotide reductase functions in the only de novo pathway for in vivo synthesis of all four deoxyribonucleoside triphosphates. The mammalian enzyme is composed of two subunits; the M1 protein (89 kilodaltons) has distinct binding sites that are involved in the allosteric regulation of enzyme activity, whereas the M2 subunit (55 kilodaltons) contains a hydroxycourea-sensitive site (1, 2). Functionally, both subunits are necessary for catalytic activity. Ribonucleotide reductase is under complex allosteric feedback control, and it has been suggested that this could provide a self-regulated flow of dNTPs for DNA synthesis (Fig. 1) (3). This model proposes activation by ATP for pyrimidine diphosphate reduction, by dTTP for guanosine diphosphate reduction, and by dGTP for adenosine diphosphate reduction. Feedback inhibition is also proposed in this model—dTTP inhibits pyrimidine biosynthesis, dGTP inhibits pyrimidine and its own biosynthesis, and dATP inhibits reduction of all four nucleoside diphosphates (4–8).

Cells synchronized in different phases of the cell cycle might reflect this allosteric regulation of their dNTP pools. Thus, during periods of active DNA synthesis (S phase), pools of dCTP and dTTP might be relatively high, whereas dATP and dGTP might be lower; during the G1 and G2 phases of the cell cycle, the opposite might occur. In addition, if allosteric regulation were the sole mechanism responsible for cell-cycle-specific alterations in dCTP production, no significant changes in ribonucleotide reduction would be expected in synchronized permeabilized cells from specific phases of the cell cycle.

We have tested each of these predictions in wild-type and two partially characterized mutant S49 cell lines with abnormal allosteric binding sites on the M1 subunit of ribonucleotide reductase (11, 12) using dibutryl cyclic adenosine monophosphate and colcemid to generate populations enriched for three portions of the cell cycle: the G1, S, and G2/M phases.

**EXPERIMENTAL PROCEDURES**

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co's modification of Eagle's medium containing heat-inactivated 10% horse serum at 37°C in a 3% CO₂ (in air) atmosphere.

The hydroxyurea-resistant cell line was selected from wild-type S49 by culturing cells in the presence of progressively higher concentrations of hydroxyurea, an inhibitor of ribonucleotide reductase (13). This cell line will be described in greater detail in another report. Briefly, the line was selected by exposure of wild-type S49 cell to incrementally increasing concentrations of hydroxyurea from 50 μM to 1 mM over a 6-month period. The resulting line was 20-fold resistant to the cytotoxic effects of hydroxyurea. These cells were resistant to hydroxyurea inhibition and had 4-fold elevated CDP reductase activity and similarly elevated dNTP pools.

**Cell-cycle Analysis**—Experimental cell suspensions (5 ml) containing 5-10 x 10⁶ cells/ml were centrifuged and resuspended in 1-3 ml of hypotonic solution containing 0.05 mg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100 (14). After staining, cells were analyzed on an argon-laser (488 nm) cytofluorimeter designed by Shapiro (15). The distribution of cells in the G₁, S, and G₂/M phases were determined by counting 1000 cells and integrating under the left slope of the G₁ peak and the right slope of the G₂/M peak. The proportion in G₁ equals 2 x left slope/total; the proportion in G₂/N equals 2 x right slope/total; and the proportion in S phase is the total - (G₁ + G₂/M)/total.

**Determination of Deoxyribonucleoside Pools**—2.5-5 x 10⁷ cells were washed in phosphate-buffered saline, centrifuged in a microfuge, resuspended in 200 μl of ice-cold 0.1 M potassium phosphate buffer, pH 7.2, and an aliquot was counted on a Coulter model ZBI. 1.5 n perchloric acid (40 μl) was added, followed 60 s later by the addition of 80 μl of 1.2 n KOH (final pH = 7.0), and samples remained on ice for 5-10 min, followed by a centrifugation at 4°C in a microfuge. These extracts were analyzed for ribonucleotides. For deoxyribonucleotide-pool measurements, periodization of the extract with 0.02 M sodium periodate for 90 min at 37°C was performed by the method of Garrett and Santi (16). The reaction was terminated by the addition of 0.03 M thiosulfate. Extracts were analyzed on a Beckman of Garrett and Santi (16). The reaction was terminated by the addition of 0.03 M thiosulfate. Extracts were analyzed on a Beckman model 211. 1.5 n perchloric acid (40 μl) was added, followed 60 s later by the addition of 80 μl of 1.2 n KOH (final pH = 7.0), and samples remained on ice for 5-10 min, followed by a centrifugation at 4°C in a microfuge. These extracts were analyzed for ribonucleotides. For deoxyribonucleotide-pool measurements, periodization of the extract with 0.02 M sodium periodate for 90 min at 37°C was performed by the method of Garrett and Santi (16). The reaction was terminated by the addition of 0.03 M thiosulfate. Extracts were analyzed on a Beckman model 211.

**Ribonucleotide Reductase Assay**—Ribonucleotide reductase activity was measured by the conversion of CDP to deoxyCDP by permeabilized cells or partially purified cell extracts. 1.2 x 10⁹ cells were permeabilized by exposure to 1% Tween 80 by the method of Lewis et al. (19). The assay mixture included final concentrations of 28 mM Hepes, 6.6 mM ATP, 8 mM MgCl₂, 50 μM CDP, and 0.42 μCi of [¹⁴C]CDP in a final volume of 300 μl after addition of 60 μl of a 5-fold concentrated mixture. To assay the partially purified enzyme preparation, 5 nM Na²⁺ was added to the other chemicals to diminish phosphatase activity. The reaction was terminated by boiling the samples for 4 min. 6.0 mg of Crotalus atrox venom/assay was then added, and samples were incubated for 4 h at 37°C. Samples were diluted with 500 μl of H₂O, loaded onto 1 ml of borate Dowex 1 columns, and [¹⁴C]deoxyxytidine product was eluted from the column with 2-3 ml of water. Samples were then counted by liquid scintillation.

**RESULTS**

**Cell-cycle-phase Enrichment**—Flow cytometric analysis of S49 cells is shown in Fig. 2. Exponentially growing cells are

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1 D. A. Albert, manuscript in preparation.

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The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
when exposed to cyclic AMP or Bt2cAMP (20). Cells exposed observed a 50% reduction in the dCTP and dTTP pools, but pools during S phase.

Cells reversibly arrest in the G1 phase of the mammalian cell cycle when exposed to cyclic AMP or Bt2cAMP (20). Cells exposed to 500–1000 μM Bt2cAMP accumulate in G1 (about 90%) within 24 h of Bt2cAMP addition, with the remaining 10% in G2/M (Fig. 2B), with no loss in cell number or viability, as assessed by either microscopic examination or [35S]methionine incorporation into cell protein. S49 cells arrested in the G1 phase may be released from this arrest by the removal of Bt2cAMP. This release results in a maximal enrichment of S phase cells (about 60%) 24 h after Bt2cAMP removal (Fig. 2A). Colcemid (0.5 μg/ml) synchronizes S49 cells in the G2/M phases of the cycle. This arrest is maximal at 8 h (about 90%) (Fig. 2D) and subsequently results in an increasing proportion of tetraploid cells.

Nucleotide Pools—Deoxyribonucleoside triphosphate pools in synchronized populations of cells are shown in Table I. We observed a 50% reduction in the dCTP and dTTP pools, but normal dATP and dGTP pools in G1 arrested wild-type S49 cells, as compared to exponentially growing cells. In populations enriched for S phase cells, the dCTP pool was elevated, and the dTTP pool was slightly depressed, as compared to exponentially growing wild-type S49 cells. A decline in the dATP pool was observed in S phase-enriched cells (Table I, Fig. 3). In G2/M enriched wild-type S49 cells, the dCTP and dTTP pools were approximately the same as those in exponentially growing cells, whereas the dATP pool was much larger (Table I).

The mutant dGuo-L cells exhibited the same pattern of dNTPs during G1 arrest that wild-type cells did. By contrast, the dGTP pool of this dGTP-resistant mutant increased throughout S and G2/M. The dTTP pool during S phase was considerably higher than that of wild-type cells as well (Table I). This suggests that feedback inhibition by dGTP is an important control limiting the size of the dTTP and dGTP pools during S phase.

The mutant dGuo-200-1 also had low dCTP and dTTP pools in G1, similar to wild-type cells. However, there was a striking increase in all four dNTP pools during S phase which persisted through G2/M (Table I). These measurements also suggest that dATP feedback inhibition is important in limiting the dNTP pools during the cell cycle. In addition, the ATP pool varied during the cell cycle (Table I). In wild-type S49 cells arrested in the G1 phase of the cycle, the ATP pool was only about 60% of that in S phase-enriched populations, whereas the GTP pool variation was less than 10% (data not shown). Dose-titration experiments revealed no augmentation of the ATP concentration by Bt2cAMP, indicating that it does not contribute to the adenine nucleotide pool.

Ribonucleotide Reductase Activity—The activity of the enzyme ribonucleotide reductase was measured by using the substrate CDP. CDP reduction in permeabilized S49 wild-type cells paralleled the dCTP and dTTP pool sizes. As shown

### Table 1

| Wild type | G1 arrested | S enriched | G2/M enriched |
|-----------|-------------|------------|---------------|
| dCTP 34.0 ± 12.1 | G1 arrested | S enriched | G2/M enriched |
| dTTP 81.0 ± 9.3 | 17.9 ± 6.8 | 49.5 ± 3.5 | 34.2 ± 11.4 |
| dATP 42.0 ± 9.5 | 37.1 ± 6.8 | 55.0 ± 14.4 | 96.8 ± 15.2 |
| dGTP 20.2 ± 7.5 | 41.0 ± 9.1 | 31.0 ± 4.2 | 113.8 ± 6.3 |
| ATP 3454 ± 749 | 19.8 ± 5.3 | 18.5 ± 6.3 | 26.5 ± 6.4 |

* Single determination.
in Table II, CDP reductase activity declined 50% or more in G1 arrested cells, whereas S phase enriched populations equaled or exceeded the activity in exponentially growing control cultures. Colcemid-arrested G2/M cells had activity that was further increased over control cultures. Enzyme activity (per mg of protein) measured in sonicates of cell-cycle-enriched cultures was similar to the data from permeabilized cells except for the results of cells in the G2/M phase (Table II).

**Identification of the M1 Subunit of Ribonucleotide Reductase on Two-dimensional Gels—**M1 was purified approximately 100-fold according to the method of Gudas et al. (17) as briefly described under "Experimental Procedures." The final elution of M1 from the dATP-Sepharose column by ATP was performed in steps of 5, 20, 40, and 60 mM and is shown in Fig. 4. Contaminating proteins were eluted at lower ATP concentrations with some M1. In the 20 mM ATP-elution fraction, M1 can be seen in relationship to other proteins that remain by comparison of a two-dimensional gel of the elution fraction with one showing a [35S]methionine-labeled whole-cell extract (Fig. 5). At 60 mM ATP, only a horizontal triplet (the third density is less intense and is poorly seen) was eluted. When aliquots of eluted protein are recombined with the dextran blue Sepharose column flow-through (which contains the M2 subunit but no M1 subunit), CDP reductase activity can be reconstituted. This activity corresponds to the intensity of M1 on the gels. When fractions of purified ribonucleotide reductase M1 subunit were mixed with [35S]methionine-labeled cell extracts and run on a two-dimensional gel, the Coomassie blue-stained purified M1 could be aligned with corresponding radiolabeled spots.

**Identification of the M2 Subunit of Ribonucleotide Reductase on Two-dimensional Gels—**The M2 subunit of ribonucleotide reductase has been tentatively identified on two-dimensional gels (Fig. 6) by using a hydroxyurea-resistant S49 cell line. A single spot demonstrated increased density which corresponded to the degree of hydroxyurea resistance.

### Table II

*CDP reductase assay as described under "Experimental Procedures"

|                  | Exponentially growing | G1 enriched | S enriched | G2/M enriched |
|------------------|-----------------------|-------------|------------|---------------|
| A. Permeabilized cells |                       |             |            |               |
| Wild type        | 22.0                  | 5.8         | 24.5       | 33.0          |
| dGuo-L           | 19.0                  | 8.3         | 34.5       | 45.0          |
| dGuo-200-I       | 41.25                 | 15.8        | 56.0       | 71.0          |
| B. Cell extracts |                       |             |            |               |
| Wild type        | 207                   | 10          | 112        | 40            |

**Fig. 4. Purification of the M1 subunit of ribonucleotide reductase.** Panels A–D are Coomassie Blue-stained two-dimensional gels of 5, 20, 40, and 60 mM ATP elution fractions from a dATP-Sepharose column, respectively. The horizontal triplet (the third density of which is poorly seen) is M1, as indicated by the arrows. The relative enzyme activity (in rpm) for the fractions represented by panels B–D when mixed with a fixed amount of M2 were 740, 770, and 610, respectively.

**Fig. 5. Relationship of M1 to other cell proteins on two-dimensional gels.** A 20 mM ATP elution fraction is enriched for M1 but also contains contaminating cell proteins, including actin (Panel A). Comparison of a two-dimensional gel of this fraction (Panel A) with one of a [35S]methionine-labeled whole cell extract (Panel C) demonstrates the location of M1 by molecular weight, pl, and in relationship to the major cell protein actin. The location was further confirmed by mixing purified M1 from a 60 mM elution fraction (Panel B) with [35S]methionine-labeled cell extract and aligning the Coomassie Blue-staining M1 with an autoradiograph of this gel. M1 is indicated by an arrow in each panel. K, kilodaltons.
size estimates of M2 and corresponded in pi and molecular weight to a spot identified as M2 in fibroblasts by Lewis and Srinivasan (21).

**Cell-cycle-enriched Two-dimensional Gels**—Using the previously described cell-cycle-phase enrichment procedures, we attempted to discern a difference in either the M1 subunit or the putatively identified M2 subunit spot in gels from [35S]methionine-labeled cultures enriched for G1, S, or G2/M phase cells (Fig. 6). Enriched cultures were labeled identically and the same amount of radioactive protein was loaded on each gel; thus, the relative spot intensity should reflect the amount of that protein in those cells. In G1-arrested cells, both the M1 and M2 spots were present, but we were unable to detect a difference in spot density when compared with S phase or G2/M phase enriched cells.

**DISCUSSION**

The regulation of deoxyribonucleoside triphosphate synthesis is closely linked to cellular-DNA synthesis. Inhibitors of ribonucleotide reductase such as hydroxyurea (22) or the feedback inhibitors dATP and dGTP decrease deoxyribonucleoside triphosphate pools and inhibit DNA synthesis (23). In addition, alterations in the normal pool sizes have been implicated in mutagenesis by potentiating mispairing (24-26). Thus, it appears that a closely regulated continuous flow of dNTPs is necessary for accurate DNA synthesis and that allosteric regulation might be involved in this regulation.

The deoxyribonucleotide pool data in G1-arrested wild-type S49 cells is consistent with allosteric control of ribonucleotide reductase in G1. dCTP, dTTP, and ATP are consistently low and dATP and dGTP are normal or elevated in G1-arrested wild-type S49 cells when compared with either exponentially growing controls, S phase enriched populations, or G2/M enriched populations. S phase enriched wild-type cultures exhibited an increase in dCTP, dTTP, and ATP consistent with activation of ribonucleotide reductase, but a decrease in dATP was less regularly observed during S phase. However, we did observe a decrease in dATP level which occurred 4 h after release from BtgcAMP-induced G1 arrest (Fig. 3). Previous studies have also shown dNTP-pool-size changes during the cell cycle. Skoog et al. (27, 28) documented increased pools of all four deoxynucleoside triphosphates during S phase, with dCTP exhibiting the largest increase. Studies in lymphocytes have shown similar results (29, 30).

We also employed two mutant cell lines: dGuo-L, a cell line heterozygous for a mutant ribonucleotide reductase that is dGTP feedback resistant, and dGuo-200-1, a cell line heterozygous for a mutant ribonucleotide reductase that is dATP feedback resistant. If dATP and dGTP were inhibitory effector molecules for deoxyribonucleoside triphosphate synthesis, then these mutant cell lines might fail to show the "inhibitory" pattern of deoxyribonucleotide pool levels that we observed in wild-type S49 cells arrested in G1. This was not the case—both mutant cell lines showed depressed pools of dCTP and dTTP and normal dATP pools in G1-arrested populations. However, a suggestion of abnormal cell cycle regulation of dNTP pools came from the S phase and G2/M phase enriched mutant cell lines. Both the dTTP and dGTP pools were elevated in dGuo-L cells suggesting a lack of dGTP feedback

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**Fig. 6.** [35S]Methionine-labeled two-dimensional gels from synchronized cultures. M1 and M2 are indicated by arrows and the enlarged insets, from G1 arrested (top panel), S phase enriched (middle panel), and G2/M enriched (bottom panel) cell cultures, respectively. Cultures from cycle enriched populations were labeled with [35S]methionine as stated under "Experimental Procedures." Labeled cells were resuspended in a small volume of lysis buffer and 1 × 10^6 cpm were loaded on each gel. Gels were exposed for 7 days each. Insets show expanded views of ribonucleotide reductase M1 and M2 subunits.
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inhibition. dGuo 200-1 cells had very elevated pools of dCTP and dTTP in S phase, suggesting a decreased ability to slow dNTP production by dATP feedback inhibition. It should be noted that the mutants are heterozygous for the mutant ribonucleotide reductase M1 subunit; thus, they might not exhibit drastically disordered cell-cycle regulation of dNTP pools.

Ribonucleotide reductase activity in the absence of allosteric effector molecules was measured by the permeabilized cell CDP reductase assay. G1 arrested wild-type S49 cells had significantly less CDP reductase activity than either exponentially growing controls or cells synchronized in S or G2/M phase (Table II). Conversely, S and G2/M phase enriched cultures showed CDP reductase activity that was greater than that measured in exponentially growing cells. These data are consistent with the results of Kucera et al. (31) and Murphree et al. (32). In contrast, Lewis et al. (19) found that, in permeabilized hamster cells, ribonucleotide reductase activity increased during S phase but declined rapidly at the end of S phase. If the activity of ribonucleotide reductase were regulated solely by allosteric effector molecules during the cell cycle, then the enzyme activity in permeabilized cells should be the same throughout the cell cycle; our data thus suggest a nonallosterically mediated increase in ribonucleotide reductase activity in S phase.

There are many possible mechanisms for a nonallosteric increase in CDP reductase activity. Five possibilities (not necessarily exclusive) that have been suggested are: 1) the compartmentalization of ribonucleotide reductase into a multienzyme complex including the enzymes necessary to process precursor nucleotides into deoxynucleotide substrates for DNA polymerase (33, 34), 2) increased quantity of the M2 subunit of ribonucleotide reductase (35), 3) nuclear translocation of ribonucleotide reductase (36), 4) changes in the endogenous hydrogen donor system (31, 37), and 5) association with the nuclear matrix (38).

We examined one of these possibilities, that an increased quantity of either the M1 or M2 subunit of ribonucleotide reductase is responsible for the increased activity observed in S phase cells. We identified M1 and tentatively identified M2 on two-dimensional gels, and then compared the intensity of these spots on gels of [35S]methionine-labeled synchronized populations in different phases of the cell cycle. The spots that correspond to the M1 and M2 subunits of ribonucleotide reductase did not change by greater than 2-fold in intensity on two-dimensional gels from [35S]methionine labeled cultures enriched for G1 phase, S phase, or G2/M phase wild-type S49 cells. Thus, we conclude that the increased ribonucleotide reductase activity seen in S phase cells compared with G1 arrested cells is not due to a large induction of the synthesis of either the M1 or M2 subunit.

Our data are consistent with previous observations on the cell-cycle control of ribonucleotide reductase activity by Eriksson and Martin (35). They noted a 6-fold increase in ribonucleotide reductase activity in S phase that appeared to be due to increased M2 rather than M1 subunit activity. Our data suggests that this may not be due to an increased quantity of M2 protein.

In summary, we have provided evidence that allosteric control of ribonucleotide reductase activity accounts for some of the observed changes in deoxynucleobisphosphate triphosphate pools during the cell cycle, especially during the G1 phase. Nonallosteric activation of the enzyme must occur in S phase, but we were unable to demonstrate induced synthesis of either the M1 or M2 subunit of ribonucleotide reductase as a source of increased activity in S versus G1 phase cells. Further study will be necessary to ascertain the mechanism by which ribonucleotide reductase activity is increased in S phase cells.

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