Activities of Thymidine Kinase and Thymine Deoxyribonucleotide Phosphatase during Growth of Cells in Tissue Culture

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Accumulating evidence indicates that the initiation of deoxyribonucleic acid synthesis is intimately associated with the appearance of elevated levels of enzymes involved in the synthesis of thymidine triphosphate. Thus, enhanced levels of such enzymes as thymidine kinase (1-11), thymidylate kinase (5-7, 12-14), thymidine diphosphate kinase (6, 7), thymidylate synthetase (10, 15, 16), and deoxyctydylate deaminase (10, 15-18) have been observed in cells and tissues undergoing rapid cellular proliferation, and in cultured mammalian cells after infection with virus. The fact that the activities of these enzymes gradually decrease to their resting levels upon cessation of deoxyribonucleic acid synthesis provides further support for the hypothesis that the enzymes are involved in processes related to cell division. Since the presence of thymidine triphosphate is required for deoxyribonucleic acid synthesis (19, 20), it has been suggested (6-10, 15, 16, 21) that these enzymes, by regulating the rate of formation of thymidine triphosphate, may play an important role in the control of deoxyribonucleic acid synthesis, and hence of cell division.

In a previous communication (22) we have reported the presence in human liver cell homogenates of enzymes that are capable of dephosphorylating the mono-, di-, and triphosphates of thymidine. Deoxyribonucleotide phosphatase activity has previously been shown in extracts of different mammalian tissues (10, 12, 15, 16, 21-30), and it has been suggested (16, 23, 24) that a correlation may exist between the cellular concentration of these catabolic enzymes and the mitotic index.

The present work was undertaken to study in more detail the ability of mammalian tissue culture cells to phosphorylate thymidine, and to dephosphorylate thymidine nucleotides, in relation to their growth rate. It was found that the levels of thymidine kinase, and of the phosphatases responsible for the breakdown of the mono- and diphosphates of thymidine, showed considerable variations during different phases of growth. The activity of the phosphatase catalyzing the cleavage of thymidine triphosphate was not similarly affected.

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1 In the Report on Enzyme Nomenclature (International Union of Biochemistry, Second Edition, 1965) thymidine kinase is listed as EC 2.7.1.21 and thymidylate kinase as EC 2.7.4.9. We have not identified the other enzymes mentioned here as being listed in the Report.

EXPERIMENTAL PROCEDURE

Cultivation of Cells and Preparation of Homogenates—All experiments were carried out with Chang human liver cells (31) grown in monolayer cultures under conditions described previously (22). Cells (10 to 15 million) were collected by trypsinization and centrifugation, washed twice with 0.9% NaCl, and suspended in 1 to 1.5 ml of ice-cold 0.01 m Tris-HCl buffer, pH 8.0. Homogenization was carried out for 90 sec in a small Potter-Elvehjem all-glass homogenizer, and the homogenate was used immediately for enzyme assay. Cell counts were carried out with an automatic particle counter.

Standard Incubation Conditions—Kinase assay was based on the procedure of Rollum and Potter (1). Tris-HCl buffer (0.2 M, 200 µl, pH 8.0), MgCl₂ (100 mM, 25 µl), ATP (100 mM, 25 µl), 3-phosphoglycerate (120 mM, 25 µl), and 200 µl (300 to 500 µg of protein) of the homogenate were equilibrated in 37-ml flat-bottomed glass tubes (2.2 cm in diameter) in a water bath at 37° for 5 min with continuous shaking. The reactions were started by adding 25 µl of 1 m thymidine-2-¹⁴C (about 50,000 cpm), and were terminated by rapid cooling in 0° and addition of 50 µl of 100% (w/v) trichloroacetic acid. The reaction mixture was then transferred to small centrifuge tubes, and the protein precipitate was removed by centrifugation. Aliquots (100 µl) of the supernatant fluid were applied to Whatman No. 1 paper strips (1.8 cm wide), and the reaction products were separated by descending paper chromatography at 22° in an isobutyric acid-ammonium-EDTA mixture (39). Rf values for thymidine, dTMP, dTDP, and dTTP were 0.75, 0.45, 0.31, and 0.21, respectively. The distribution of radioactivity on the developed chromatograms was determined as described by Fritson (33), with a Traceclab flow counter. The radioactivity recovered in dTMP + dTDP + dTTP, calculated as percentage of the total radioactivity on the chromatogram, was taken as a measure of thymidine kinase (1, 6). Deoxyribonucleotide phosphatase activity of the homogenate was measured as described previously (22). Protein was determined by the method of Lowry et al. (34), as modified for tissue culture by Oyama and Eagle (35), with bovine serum albumin as reference standard.

Chemicals—Thymidine, and the different nucleotides and deoxynucleotides used, were purchased from Sigma Chemical
Company. Thymidine-2-1*C (specific activity, 196 μC per mg) was obtained from The Radiochemical Centre, Amersham, England. All other chemicals used were commercial products of the highest purity, obtained from Merck and Company, Inc., Sigma Chemical Company, and Nutritional Biochemicals Corporation. The potassium salt of 3-phosphoglyceric acid was prepared from barium 3-phosphoglycerate (Sigma) by treatment with K₂SO₄.

RESULTS

Characterization of Thymidine-phosphorylating Enzyme in Chang Cells—Fig. 1 shows that thymidine-2-1*C is extensively incorporated into DNA of proliferating liver cells, indicating the presence in this cell type of the enzyme system needed for the phosphorylation of thymidine to the corresponding triphosphate. It appears that the rate of incorporation was dependent on both the concentration of thymidine in the medium and the time of incubation.

The results presented in Fig. 2 show that under standard incubation conditions for kinase assay, the amount of radioactivity recovered in dTMP + dTDP + dTTP was proportional to the amount of protein in the reaction mixture over the range studied, and to the time of incubation up to about 30 min. The data indicate that the procedure used gives an adequate measure of the thymidine kinase activity in the liver cell homogenate.

Thymidine kinase in extracts of different tissues has previously been reported to be inhibited by dTMP (11, 37, 38), dTDP (11, 28, 37, 38), dTTP (11, 28, 29, 37–40), dCMP (38), and dCTP (38, 40). In Fig. 3 it appears that addition of dTMP, dTDP, or dTTP to the incubation mixture markedly inhibited the phosphorylation of thymidine by the liver cell homogenates. dTDP and dTTP were found to be about equally effective in inhibiting thymidine kinase, while on a molar basis dTMP was much less inhibitory. The results indicate that the inhibition by dTDP and dTTP is not due to the action of dTMP or thymidine formed during incubation, in agreement with the views of other investigators (28, 37). The effect of dTDP might be due to the presence of an active dTDP kinase, rapidly converting dTDP to dTTP. Such a mechanism has been suggested by Ives et al. (28) to account for the inhibition of thymidine kinase by dTDP in Novikoff hepatoma cells. The comparatively small effect of dTMP might be due to dephosphorylation of the monophosphate, resulting in a dilution of the labeled thymidine (37), or to a slow conversion of the nucleotide to the di- and triphosphate stage during incubation, or to both.

No inhibition of thymidine kinase was found when the di- and triphosphates (20 mmoles per reaction mixture) of deoxyadenosine, deoxyguanosine, deoxycytidine, guanosine, or uridine were added to the incubation mixture. This finding indicates that the inhibition of thymidine kinase in Chang liver cells is specific for the thymidine nucleotides. Similar observations have been made in studies with regenerating rat liver (37) and Novikoff hepatoma cells (28).
The inhibition of thymidine kinase by deoxynucleotides has been shown to be of the competitive type, being reversed by elevated concentrations of thymidine in the case of dTTP (28, 37–39), dTDP (37), and dCTP (38). Ives et al. (28) found that inhibition of thymidine kinase by dTTP or dTDP in concentrations of 20 μmole per reaction mixture (Fig. 3) was reduced by a factor of 2 when the concentration of thymidine was doubled, whereas no significant effect of ATP was observed.

In the present study the radioactivity recovered in dTMP + dTDP + dTTP was taken as a measure of thymidine kinase activity. Usually, no more than 5% of the total radioactivity on the chromatogram was recovered in dTMP and about 3% in dTDP + dTTP. This corresponds to about 1.3 μmole of dTMP formed and about 0.5 μmole of dTDP + dTTP formed per reaction mixture under standard incubation conditions. From Fig. 3 it appears that these concentrations of the nucleotides would not be expected to inhibit the activity of thymidine kinase significantly, in agreement with the results shown in Fig. 2.

**Fig. 4 (left).** Dephosphorylation of thymidine nucleotides (A) and phosphorylation of thymidine (B) in relation to cell growth (C). Glass bottles (150 ml) were inoculated with 15 ml of a suspension containing 10 million cells and incubated at 37° in a water bath. The experiments were started at zero time and every 24 hours, counted, and homogenized as described in “Experimental Procedure.”

**Fig. 5 (right).** Dephosphorylation of thymidine nucleotides (A) and phosphorylation of thymidine (B) in relation to cell growth (C). Conditions were as described in the legend to Fig. 4, except that the experiments were started 48 hours after inoculation of the cultures and the medium was renewed at Point a with medium lacking glutamine and at Point b with medium containing glutamine. ▲, dTMP phosphatase; ▲, dTDP phosphatase; ☼, dTTP phosphatase.

In the above experiment the changes in cell growth occurred at a relatively slow rate. It was desirable to measure the activity of thymidine kinase and of the phosphatases responsible for the cleavage of thymidine nucleotides during different phases of cell growth. The results of such measurements are shown in Fig. 4. It appears (Fig. 4B) that the activity of thymidine kinase gradually increased during the lag and early logarithmic phase of growth, reaching a maximum after 2 days and remaining elevated during the late portion of the rapid growth phase. A decrease in the level of thymidine kinase occurred between the 4th and 5th day of cultivation, the period in which the cells entered the stationary phase. The activity of deoxynucleotidase phosphatase declined slightly during the lag phase (A). Thereafter a rise in the levels of the phosphatases responsible for the dephosphorylation of dTMP and dTDP occurred. In contrast, the activity of the phosphatase catalyzing the cleavage of dTTP remained constant throughout the growth cycle.

In the above experiment the changes in cell growth occurred at a relatively slow rate. It was desirable to measure the activity of thymidine kinase and of deoxynucleotidase phosphatase under conditions of more rapid changes in the rate of cell proliferation. This was achieved by replacing the medium of exponentially growing cultures by fresh medium lacking the essential amino acid L-glutamine (Fig. 5C). It appears that the growth rates of the cell populations rapidly decreased, and after about 30 hours cell multiplication had completely ceased. Replenishment of the medium after 2 days by medium containing glutamine was followed by a new period of rapid cell multiplication.

The results of the phosphatase (Fig. 5A) and kinase (Fig. 5B) assays are essentially in accordance with the data presented in Fig. 4. Thus, the activity of thymidine kinase declined rapidly during the period when the rate of cell growth was decreasing. Also, a rise occurred in kinase level concomitantly with the onset of rapid cell proliferation. The activity of deoxynucleotidase phosphatase responsible for the dephosphorylation of dTMP and dTDP gradually increased during the logarithmic phase of growth and the time when the cells were cultivated in the absence of glutamine. Subsequently, a marked fall occurred in the levels of these enzymes coincident with the initiation of cell multiplication. No significant changes in the activity of dTTP phosphatase were observed.

Obviously, differences in the concentration of thymine deoxynucleotidase phosphatase in the cell homogenates might affect the measurements of thymidine kinase by changing the rate of dephosphorylation of the nucleotides formed in the kinase reaction. Whether the variations here observed in thymidine kinase activity during proliferation of the liver cells may in part be accounted for by the changes in hydrolysis of dTMP and dTDP by phosphatase cannot be decided from the present data. It has been shown by Beltz (10) with preparations of normal and regenerating rat liver that addition of fluoride, which inhibits deoxynucleotidase phosphatase of rat liver (10, 15, 16, 23), did not influence the results of the deoxynucleoside kinase assay. In contrast, Behki and Morgan (21) found with extracts of regenerating rat liver that fluoride stimulated the phosphorylation of thymidine, presumably by inhibiting selectively the breakdown of thymidine monophosphate. Because of the failure of fluoride to inhibit thymine deoxynucleotide phosphatase of Chang liver cells (22), similar studies could not be carried out with our enzyme preparations.
DISCUSSION

The data reported in the present paper indicate that the ability of Chang human liver cells to phosphorylate thymidine to the corresponding nucleotides is related to their growth rate. Thus, elevated levels of thymidine kinase were found (Figs. 4B and 5B) in homogenates of rapidly proliferating cells, as compared to the levels found in homogenates of nonproliferating cells. This is consistent with the findings of previous investigators (1–3, 6–8, 10) that thymidine kinase is elevated in mitotically active tissues. The present results provide further support for the view (6, 8) that thymidine kinase may play an important role in the control of deoxyribonucleic acid synthesis and tissue growth.

Marked variations during different phases of growth were also observed in the activity of deoxyribonucleotide phosphatase responsible for the cleavage of the mono- and diphosphates of thymidine. Obviously, the levels of these catabolic enzymes may affect the intracellular pool of thymidine nucleotides. Thus, a decrease in the enzyme activities may presumably favor the accumulation of precursors required for deoxyribonucleic acid synthesis. Interestingly, in the present study a significant fall occurred (Fig. 5A) in the levels of dTMP and dTDP phosphates coincident with the initiation of rapid cell multiplication, whereas a concomitant rise occurred (Fig. 5B) in kinase level. The observed changes in the phosphatase levels are in the direction expected if these enzymes are involved in the initiation and control of deoxyribonucleic acid synthesis. Deoxyribonucleotide phosphatase activity has previously been shown to be significantly depressed in regenerating (10, 15, 16, 23) and in embryonic (23) rat liver, as compared to normal adult liver. A decrease in deoxynucleotidase has also been observed (23, 24) in rat liver hepatomas during the process of carcinogenesis.

The finding (Figs. 4A and 5A) that the activity of the phosphatase responsible for the dephosphorylation of dTTP was not affected by changes in the rate of cell growth seems to rule out a role for this enzyme in the control of cellular production of deoxyribonucleic acid. It also supports our previous suggestion (22) that the cleavage of the different phosphates of thymidine is not catalyzed by the same phosphatase.

SUMMARY

The relationship between growth rate of human liver cells (Chang) in tissue culture and the activity levels of thymidine kinase, and of thymine deoxyribonucleotide phosphatase, was investigated by measuring enzyme activities during different phases of growth.

Elevated levels of thymidine kinase were found in homogenates of cells in the rapid phase of growth, as compared to the levels found in homogenates of cells in the stationary phase. The activity of deoxyribonucleotide phosphatase responsible for the dephosphorylation of the mono- and diphosphates of thymidine showed considerable variations during cell proliferation. A marked decrease occurred in the levels of these catabolic enzymes concomitant with the initiation of rapid cell multiplication. In contrast, the activity of the phosphatase catalyzing the cleavage of thymidine triphosphate was not affected by changes in the rate of cell growth.

The data indicate that a correlation exists between the growth rate of the liver cells and the activities of thymidine kinase and of deoxyribonucleotide phosphatase hydrolyzing the mono- and diphosphates of thymidine. The results suggest a role for these enzymes in the control of deoxyribonucleic acid synthesis and cell growth.

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