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INCREASED CONCENTRATION OF THYMIDINE KINASE IN RAT HEPATOMAS

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Thymidine kinase was purified to near homogeneity by affinity chromatography from cytosol fraction of rat hepatoma 3924A. The enzyme had a Mr of 81,000 and was composed of two subunits of Mr = 44,000. Antiserum made against it neutralized the activities of thymidine kinase from both rat livers and hepatomas. Neutralization studies with the antiserum revealed that hepatic transformation resulted in 4-, 15- and 25-fold increase in the amount of cytosol thymidine kinase in hepatomas 16, 7787 and 3924A of slow, medium and fast growth rate, respectively.

The activity of thymidine kinase (TK; EC 2.7.2.21) was markedly elevated in the cytosol fraction of a wide spectrum of chemically-induced, transplantable rat hepatomas and the increase positively correlated with tumor growth rate (1). It was of interest to know whether the elevated TK activity reflected an increase in newly synthesized TK protein or activation of pre-existing molecules.

In this communication, we report the purification of TK from the cytosol fraction of a fast-growing rat hepatoma and the immunological evidence indicating that the elevated TK activity in rat hepatomas was due to a concordant increase in the concentration of thymidine kinase protein.

MATERIALS AND METHODS

Nucleosides and nucleotides were obtained from Sigma. [^{14}C]-thymidine was purchased from New England Nuclear. CH-Sepharose and Sephacryl S-200 were products of Pharmacia. Carbodiimide and calcium phosphate gel were from Bio-Rad. Palladium on carbon was from Aldrich. Goat anti-rabbit IgG was from Cappel Laboratories.

Hepatomas: Hepatomas 16 and 7787 were maintained in male Buffalo rats. Hepatoma 3924A was carried in male ACI/N rats. Normal livers from Buffalo and ACI/N rats were used as controls.

Tissue homogenization and cellular fractionation: Tissues were homogenized in 4 volumes of extraction buffer (50 mM Tris-HCl, pH 7.6, 6 mM 2-mercaptoethanol,25 mM KCl, 2.5 mM $MgCl_2$, 0.25 M sucrose, 0.5 mM phenylmethylsulfonylfluoride, and 2 mM thymidine).[2] The crude homogenates were centrifuged at 9,500

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x g and 105,000 x g, and the resultant supernatant, which contained soluble proteins present in cytosol fraction, was used to assay enzyme activities and for further purification. All purification steps were carried out at 4°C.

Partial purification: Following removal of nucleic acids by precipitation with 1% streptomycin sulfate, proteins in cytosol were precipitated with ammonium sulfate (35% saturation). The precipitates were resuspended in 1/3 the original volume in extraction buffer plus 2 mM of thymidine, and absorbed with calcium phosphate gel (1 mg gel/mg protein) for 15 min. After removal of gel by centrifugation, proteins were reprecipitated with ammonium sulfate. The precipitates were taken up in 1/10 the original volume in buffer A (25 mM Tris-HCl, pH 7.6, 6 mM 2-mercaptoethanol and 0.5 mM ATP) and dialyzed overnight against 100 volumes of buffer A.

TK assay: The activity of TK was assayed as described (1), using (^{14}C)-thymidine as substrate. One unit of TK activity is defined as the amount of the enzyme that converts one nmol of thymidine to thymidine monophosphate (TMP) per hour. Protein concentrations were determined by Bio-Rad protein assay method.

Preparation of affinity gel: A derivative of thymidine was covalently linked to agarose by the procedure developed by Kowal and Markus (2) and modified by Lee and Cheng (3). Briefly, p-nitrophenyl thymidine 3'-monophosphate was reduced to p-aminophenyl thymidine 3'-monophosphate in a hydrogenation reaction catalyzed by palladium on carbon at 1 atm of hydrogen. The reduced compound was covalently linked to the carboxyl group of CH-Sepharose in a coupling reaction catalyzed by water-soluble carbodiimide. The uncoupled carboxyl groups were blocked by excess galactosamine. The coupling efficiency was approximately 60% and yielded 4.7 μmol of the derivative bound per ml of gel.

Polyacrylamide and SDS-polyacrylamide gel electrophoresis: Electrophoresis of TK in 5% polyacrylamide gels under non-denaturing conditions was performed essentially as described by Kit et al. (4). Gels were sliced and assayed for TK activity. Relative mobility of TK to tracking dye was determined. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of TK was performed in a 12.5% gel slab as described by Laemmli (5). Proteins were visualized by Coomassie Brilliant Blue staining.

Molecular weight estimation by gel filtration chromatography: A Sephacryl S-200 column was employed. Phosphorylase B, bovine serum albumin, ovalbumin and chymotrypsinogen A were used as molecular weight standards. Elution buffer contained 50 mM Tris-HCl, (pH 7.6), 6 mM 2-mercaptoethanol, 0.1 M KCl, and 0.5 mM thymidine. TK activity in each fraction was determined. The molecular weight of TK was estimated from calibration curve prepared from standards.

Antiserum preparation and neutralization assay: A total of 1 mg of purified TK was used in three doses at four-week intervals. The immunogen was administered intradermally into New Zealand white rabbits at multiple sites on the back. Ten days after the last inoculation, antiserum was collected and partially purified by ammonium sulfate precipitation. The antiserum was stored at -20°C in 50% glycerol/phosphate buffered saline (PBS). For neutralization of TK activity, two-fold serial dilutions of the antiserum were made in PBS. Ten microliters of cytosols prepared from liver and hepatomas were incubated with 90 μl of the serum dilutions at 4°C for one hour. After immunoprecipitation with goat anti-rabbit IgG, the mixture was centrifuged at 15,000 x g for 5 min. The upper one-half of the mixture was removed and assayed for residual TK activity. After subtracting backgrounds from controls containing corresponding serum dilutions, the percentage of residual TK activity was determined, using the activity in the corresponding sample that was not treated with antiserum as 100%.

RESULTS AND DISCUSSION

Purification of TK by affinity chromatography: After calcium phosphate absorption, the partially purified TK preparation was applied to the affinity column in buffer A. The column was washed with Tris buffers of increasing strength (50 mM, 100 mM and 250 mM). The majority (> 90%) of TK was eluted with addition of 10 mM thymidine in 250 mM Tris buffer. A minor peak of activity (< 10%) was eluted when buffer strength was increased to 500 mM (Fig. 1). The fractions in the major peak were pooled and further purified by a second affinity column. After two cycles of affinity chromatography, a purification of over 20,000 fold with a recovery greater than 40% of the original activity was usually achieved. Table 1 summarizes a typical purification procedure.

Molecular characterization of purified TK: The purity of TK obtained at the last step of purification (Fraction 5) was examined by SDS-polyacrylamide gel electrophoresis. Figure 2 depicts Coomassie Blue staining patterns of polypeptides present in Fraction 4 (from first affinity column; Fig. 2B) and Fraction 5 (from second affinity column; Fig. 2C). It is clear that after two cycles of

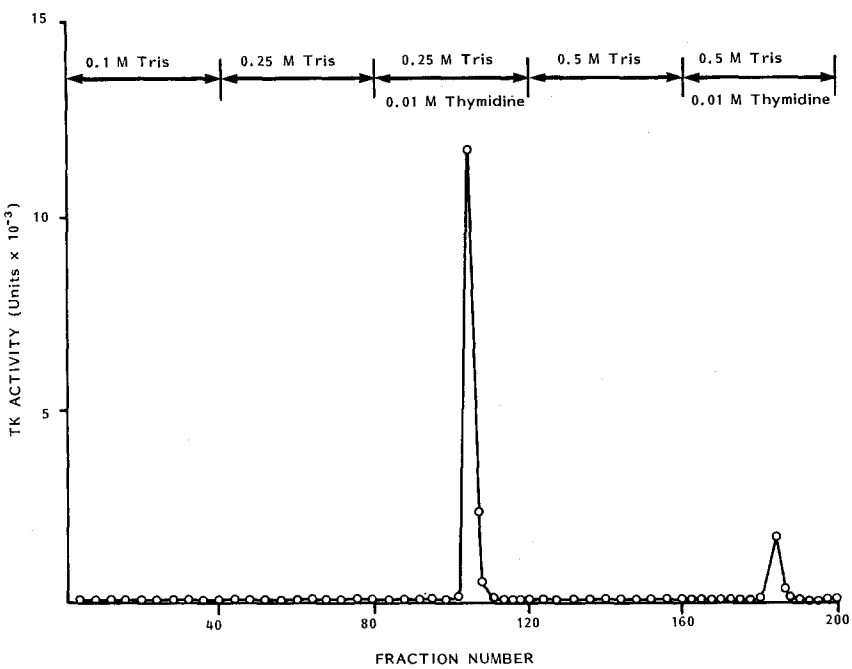

Fig. 1. Elution profile of TK from affinity column of [p-aminophenyl thymidine 3'-monophosphate]-CH Sepharose.

Table 1: Purification of thymidine kinase from hepatoma 3924A

Fraction	Protein (mg)	Activity (units)[a]	Units/mg protein	Purification (-fold)	Recovery (%)
1. 105,000 x g supernatant	10,620	122,400	11.5	1	100
2. Streptomycin/ ammonium sulfate	2,780	76,700	28.0	2	63
3. Calcium phosphate/ ammonium sulfate	680	68,550	101.0	9	56
4. First column peak fractions	8.31	59,070	7,508	660	49
5. Second column peak fractions	0.22	53,242	237,680	20,570	44

[a] 1 unit = 1 nmol of TMP formed at 37°C in 60 min.

affinity chromatography, TK present in the cytosol fraction of hepatoma 3924A was purified to near homogeneity. The apparent molecular weight of its monomer was estimated to be 44,000 daltons. The molecular weight of TK in its native state

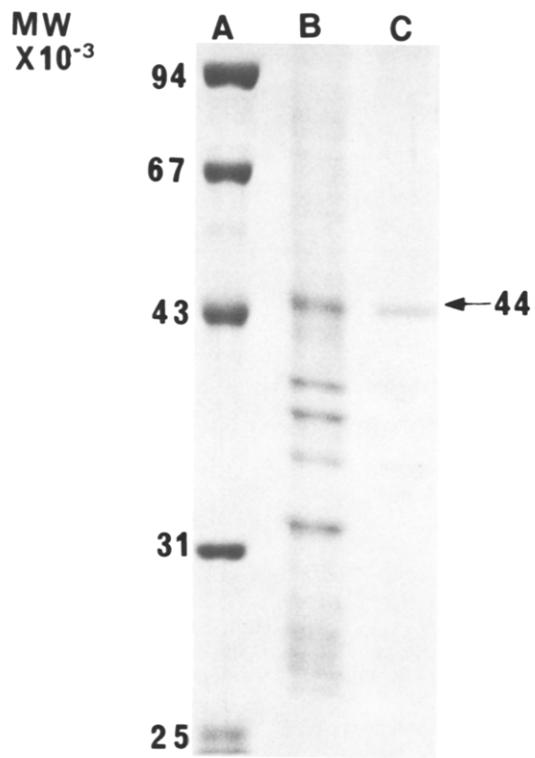

Fig. 2. SDS electrophoretic analysis of polypeptides present in peak TK fractions eluted from affinity column. Track A: protein standards. Track B: TK from first column (Fraction 4, Table 1). Track C: TK from second column (Fraction 5, Table 1). Electrophoresis was performed in a 0.1% SDS-12.5% polyacrylamide slab gel.

was estimated to be 81,000 daltons by gel filtration chromatography, The figure was in accord with those estimated for regenerating rat liver (6,7). It was concluded that cytosol TK of hepatoma 3924A was composed of two subunits of equal size.

The presence of cytoplasmic and mitochondrial isozymes of thymidine kinase has been demonstrated in many mammalian cells. The isozymes can be easily distinguished by their relative electrophoretic mobilities (R_f) in polyacrylamide gels under nondenaturing conditions. In 5% polyacrylamide gels, the R_f values for cytosol TK and mitochondrial TK are approximately 0.2 and 0.6, respectively (5). TK purified from the cytosol of hepatoma 3924A (Fraction 5) was subjected to electrophoresis in polyacrylamide gels in parallel with the crude homogenate and the crude cytosol (Fraction 1, Table 1). Fig. 3A depicts the electrophoretic characteristics of TK present in the crude homogenate. Two major peaks of activity were discerned, the first with an R_f value of 0.16 and the second with an R_f of 0.57. In the crude cytosol (Fraction 1) preparation (Fig. 3B), the major TK activity migrated with an R_f value identical to that of peak I in the crude homogenate. There remained some activity in peak II area, presumably due to leakage of TK from mitochondria during homogenization. Fraction 5 (Fig. 3C), on the other hand, migrated as a single peak with an R_f of 0.16 and was virtually free of peak II material.

Neutralization of TK activities in rat livers and hepatomas: Anti-TK antiserum was serially diluted and tested for its ability to neutralize the activities of TK present in the cytosols of rat livers (both ACI/N and Buffalo strains), and of hepatomas 16, 7787 and 3924A. The residual activity was determined as described in the Methods section. Preliminary studies revealed that the antiserum against cytosol TK of hepatoma 3924A effectively neutralized the activities of its counterparts from livers of ACI/N rat (control for hepatoma 3924A) and Buffalo rat (control for hepatomas 16 and 7787). The kinetics of neutralization of TK activities in the liver and hepatomas (Fig. 4) indicate that the antiserum against cytosol TK of hepatoma 3924A recognized those of hepatomas 16 and 7787 as well. These results also suggest (1) that cytosol TK's from ACI/N and from Buffalo rat liver were closely related; and (2) that hepatic transformation did not cause

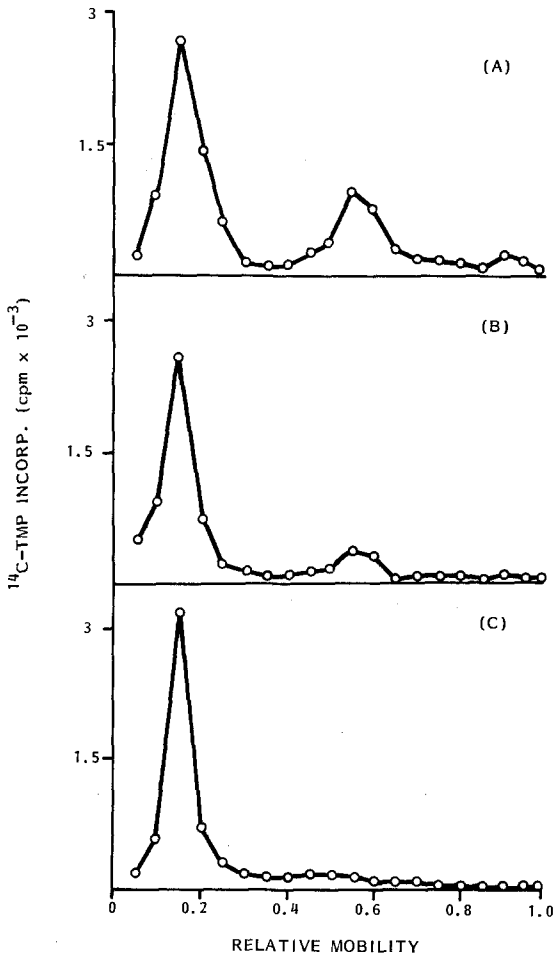

Fig. 3. Electrophoretic patterns of TK from hepatoma 3924A. Approximately equal amounts of TK activity from each sample were analyzed in 5% polyacrylamide gels. The conditions of electrophoresis, extraction and assay of TK were described in the text. (A) crude homogenate; (B) 105,000 x g supernatant (cytosol, Fraction 1); (C) purified TK (Fraction 5).

gross structural alterations in cytosol TK of the hepatomas which might affect the antigenicity of the enzyme. From Figure 4, the titer of the antiserum needed to inhibit one-half of TK activity in each sample was determined. The results are compiled in Table 2 together with growth characteristics and TK activities of these hepatomas. It is evident that hepatic transformation induced 5-, 16- and 30-fold increases in cytosol TK activity in hepatomas 16, 7787 and 3924A, respectively. Comparison of anti-TK titers revealed that 4-, 15- and 25-fold excess of antiserum was required to neutralize 50% of cytosol TK activity in

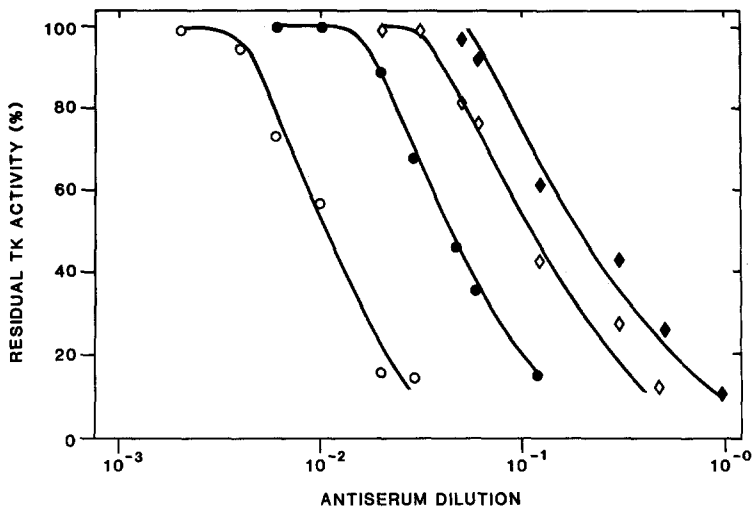

Fig. 4. Neutralization kinetic curves of TK from different hepatomas with anti-TK antiserum. Experimental conditions were detailed in Methods section. o—o—o Liver (ACI/N & Buff. rats); ●—●—● Hepatoma 16; ◇—◇—◇ Hepatoma 7787; ◆—◆—◆ Hepatoma 3924A.

hepatomas 16, 7787 and 3924A. These two sets of figures agree well and suggest that the elevated cytosol TK activity in rat hepatomas of different growth rates was a result of a concordant increase in the concentration of the enzyme protein upon neoplastic transformation.

The molecular basis of the altered TK gene expression in rat hepatomas has yet to be established. Recent studies on the expression of α-fetoprotein and albumin genes in rat hepatomas indicate that the changes in the levels of these

Table 2: Neutralization titers of anti-TK antiserum for different rat hepatomas

Tissues	Tumor growth rate: wks to reach 1.5 cm diameter	TK activity nmol/hr/mg protein	TK activity % of control	50% neutralization titers 1/serum dilution	50% neutralization titers % of control
Liver controls	-	0.41	100	6.2×10^{-3}	100
Hepatoma 16	41.0	1.93	471	25.3×10^{-3}	408
7787	27.0	6.51	1,588	90.3×10^{-3}	1,456
3924A	4.3	12.21	2,978	151.7×10^{-3}	2,447

two serum proteins occur at the transcriptional level (8,9,10). Whether such a mechanism leads to the increased amounts of TK in rat hepatomas remains to be explored. Experiments are currently underway to investigate the TK mRNA activities in liver and hepatomas in an attempt to understand the molecular events which lead to the heritable alteration of TK gene expression.

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