Regulation of Succinyl Coenzyme A:Acetoacetyl Coenzyme A Transferase in Rat Hepatoma Cell Lines

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ABSTRACT

The regulation of succinyl-CoA:acetoacetyl-CoA transferase (CoA transferase) has been studied in 8 rat hepatoma cell lines. Compared with normal rat hepatocytes, which have almost nondetectable activity of the enzyme, the hepatoma cell lines have a wide range of expression of CoA transferase activity, from as low as 45 nmol/min/mg to as high as 960 nmol/min/mg. Western blotting showed that the different levels of CoA transferase activity were due to differing amounts of the enzyme in the cells. This was further attributed to the varying amounts of the enzyme synthesized in the cells as monitored by 14C-methionine labeling followed by immunoprecipitation. To study further the differential expression of CoA transferase in the hepatoma cell lines, the relative quantity of functional CoA-transferase mRNA in the cells was measured by in vitro translation. The results showed that the levels of functional CoA transferase mRNA detected were consistent with the differences in the enzyme activity in the cells. Since CoA transferase is the key enzyme responsible for the utilization of ketone bodies as an alternative energy source, the expression of CoA transferase in hepatoma cells may play a role in energy production.

INTRODUCTION

Ketone bodies (D-β-hydroxybutyrate and acetoacetate) are largely produced by mammalian liver and consumed by nonhepatic tissues as energy substances and lipogenic precursors (1–3). The key enzyme responsible for consumption of ketones for energy production is CoA transferase,4 which catalyzes the rate-limiting step converting acetoacetate to acetoacetyl-CoA in the presence of succinyl-CoA (4–6). In the normal state, CoA transferase is expressed at high levels in many tissues except liver (7–9). Therefore, the existence of CoA transferase in any given tissue determines the capability of this tissue to utilize ketone bodies. Because the major role of ketone bodies is to supply an alternative substrate to glucose for nonhepatic tissues (10), the regulation of CoA transferase serves as an additional control of energy metabolism in nonhepatic tissues.

It has been shown that CoA transferase can be differentially regulated in many tissues (11–13). Changes of CoA transferase activity can also occur in liver. Normal hepatocytes have very low levels of the enzyme. However, transformed hepatocytes, as well as a variety of hepatoma cell lines, express CoA transferase (14). Thus hepatoma cells can mimic the behavior of nonhepatic cells in terms of ketogenic utilization. Although it has been demonstrated that CoA transferase activity is increased to different extents in different rat hepatoma cell lines (15–17), the mechanism for this differential expression has not been determined.

In this article, we studied the basis underlying the increase of CoA transferase activity in rat hepatoma cell lines. By quantitating enzyme content, biosynthesis, and performing in vitro translations, our experiments indicate that variations in CoA transferase activity of different hepatoma cell lines are due to different amounts of enzyme protein, which are correlated with the variations in the levels of functional CoA transferase mRNA in the corresponding cell lines.

MATERIALS AND METHODS

Reagents. Chemicals are reagent grade unless otherwise specified. Solutions were prepared in deionized water. Goat anti-rabbit IgG-peroxidase conjugate, collagenase, 14C-methionine solution (200×), aprotinin, protein-A Sepharose CL-4B, and 4-chloro-l-naphthol were purchased from Sigma (St. Louis, MO). Gauadinium thiocyanate was from Fluka (Buffalo, NY). Adjuvant was from RIBI, Inc. (Hamilton, MT). Heat-inactivated fetal bovine serum was from Hyclone Company (Logan, UT). DME was from K.C. Biologicals, Inc. (Lenexa, KS). Methionine-free DME was from Gibco, Inc. (Grand Island, NY). 14C-Methionine (for biosynthesis, 1153 Ci/mmole) was from ICN Radiochemicals (Irvine, CA). Nuclease-treated rabbit reticulocyte lysate from Promega (Madison, WI). 14C-Methionine (in vivo translation grade, 1209 Ci/mmole) and Enlightening rapid autoradiography enhancer were from Du Pont-New England Nuclear (Boston, MA).

Hepatoma Cell Lines and Culture Conditions. HTC cells were originally from Dr. E. B. Thompson, National Cancer Institute. JM2 cells were from Dr. Michaelpoulos, Duke University Medical Center. The McA-RH7777 and H4-II-E3C lines were originally obtained from the American Type Culture Collection (Rockville, MD). RLT-2M, RLT-3C, RLT-5G, and RLT-9F are hepatoma cell lines established in our laboratories from a hepatocellular carcinoma induced by 1-day-old Sprague-Dawley rats with diethylnitrosamine followed by weaning by phenobarbital promotion (18). The hepatocyte-specific marker tyrosine aminotransferase was utilized to identify these cell lines were derived from hepatocytes. Cells were routinely grown under an atmosphere of 5% CO2 and 95% air as monolayers at 37°C in 25-cm2 tissue culture flasks containing 5.0 ml of DME supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, and 1% nonessential amino acids.

Animals. Adult Sprague-Dawley rats (200–250 g) were from a breeding colony maintained at the University of Alabama central animal facility. Adult female New Zealand white rabbits were from Myrtle’s Rabbity (Thompson Station, TE). Animals had access to tap water and food ad libitum.

Preparation of Mitochondria from Rat Hepatocytes and Hepatoma Cells. Hepatoma cells were harvested by treatment with 0.025% trypsin in phosphate-buffered 0.85% NaCl (pH 7.2) followed by pelleting the cells at 400 × g for 5-min centrifugation. Normal rat hepatocytes were prepared by the perfusion procedure of Berry and Friend (19) with the modification of Kreamer et al. (20). The cells were homogenized with a Wheaton Overhead Stirrer at low speed in 1-g tissue/5-ml homogenizing buffer (0.25 m sucrose buffered with 50 m manganese trifluoracetate, pH 7.4/5.5 M EDTA). The procedure of mitochondria isolation was modified from the method of Bustamante et al. (21).

Determination of Hepatoma Cell Growth Rate. Cells of each line (4 × 105) were seeded into 25-cm2 flasks. Cells were counted from triplicate flasks every day until day 7. Doubling times were determined from the linear parts of the exponential growth curves.

Enzyme Activity Assays. Protein concentration was measured by the biocinchoninic acid protein assay (22) with bovine serum albumin as control of energy metabolism in nonhepatic tissues.

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<3> The abbreviations used are: CoA transferase, succinyl-CoA:acetoacetate-CoA transferase (EC 2.8.3.5); DME, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
protein standard. Mitochondrial membranes were disrupted by sonication of the mitochondrial suspension diluted in 10 mM Tris-HCl (pH 7.8)/1 mM EDTA with a Fisher Sonic Dismembrator Model 300. CoA transferase activity was determined as described by Williamson et al. (5), by measuring the rate of acetoacetyl-CoA disappearance at 303 nm in the presence of sonicated mitochondria and succinate. The activity of malate dehydrogenase was assayed essentially as described previously (23).

**Imunochemical Quantitation.** Rat brain CoA transferase was purified to homogeneity using the procedure of Russell and Patel (24). Polyclonal antibodies against the purified enzyme were raised in rabbits and the antisem to CoA transferase was prepared according to standard methods (25). Immunochemical quantitation (Western blotting) of CoA transferase in the mitochondria was done essentially as described (26, 27).

**Radioactive Labeling.** The labeling procedure was performed essentially as described (28, 29). Hepatoma cells or freshly isolated hepatocytes were washed twice with PBS and preincubated at 37°C at 1 x 10⁶ cells/ml in medium without [35S]methionine for 2 h. Labeling was stopped by replacing the radioactive medium with 5 ml of PBS containing 0.1 mM [35S]methionine and 50 mM HEPES. Cells were labeled for 60 min at 37°C with 200 nCi/ml. Incorporation proceeded for 2 h. Labeling was stopped by replacing the radioactive medium with 5 ml of PBS containing 0.1 mM [35S]methionine. Labeled cells were collected and lysed with 4% SDS in a volume of 100 μl in a boiling water bath for 5 min.

**In Vitro Translation.** Normal rat hepatocyte and hepatoma RNA were prepared by the method of Chirgwin et al. (30). RNA preparations were judged to be intact by agarose electrophoresis with visualization of the 18S and 28S rRNA bands (31). The RNA was translated in vitro as described by Pelham and Jackson (32) using a micrococcal nuclease-treated rabbit reticulocyte lysate translation system incorporating L-[35S]methionine at a concentration of 1.0 μCi/μl reaction volume. Translation reactions were stopped by adding SDS to final 4% (w/v) in the reaction mixture and boiled in water bath for 3 min.

**Immunoprecipitation.** Radioactive-labeled total cell lysates or in vitro translation products were diluted with an equal volume of PBS to reduce SDS concentration to 2% followed by adding 4 volumes of dilution buffer (2.5% Triton X-100; 190 mM NaCl; 60 mM Tris-Cl, pH 7.4; 6 mM EDTA; and 10 units of aprotinin). Aliquots were taken from the diluted lysates to determine trichloroacetic acid precipitable counts. Immunoprecipitation was carried out as described by Anderson and Blobel (33).

**SDS-PAGE and Fluorography.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (34) in 0.1% SDS-Tris/glycine buffer on 9% separating gels. Proteins were visualized in the rat hepatoma cell lines is slightly less than M, 55,000, which is the molecular weight of the rat brain enzyme (Fig. 1, lane 2). CoA transferase in normal hepatocyte mitochondria was not detectable by Western blotting (Fig. 1, lane 1). In the cell lines that have the least activity, RTL-3C, McA-RH7777, and H4-II-EC3 (Fig. 1, lanes 8–10) there is an immunodetectable band of slightly higher molecular weight than the major band. We do not know if this higher molecular weight band is a precursor of the lower molecular weight species. It should be noted that the cell line H4-II-EC3, which has the lowest CoA-transferase activity, expresses only the high-molecular-weight band. For purposes of quantification, all immunoreactive bands on the Western blots were scanned to determine relative levels of CoA transferase. These data are given in Table 1.

In order to further demonstrate the specificity of our CoA-transferase antiserum, we immunoprecipitated CoA transferase activity from sonicated brain and rat hepatoma cell line JM-2 mitochondria. Following incubation with either preimmune serum or CoA-transferase antibodies, protein A-Sepharose was added, and the antibody solid-phase complex was isolated by centrifugation. The supernatant was then assayed for CoA transferase activity. Preincubation with preimmune serum and protein-A Sepharose resulted in a loss of less than 10% of the activity from both brain and JM-2 sonicated mitochondria. When CoA-transferase antiserum and protein A-Sepharose were used, greater than 90% of all activity was eliminated from both JM-2 mitochondria and rat brain mitochondria (data not shown).

**RESULTS**

**CoA Transferase Activity in Hepatoma Cell Lines.** The activity of succinyl-CoA:acetoacetyl-CoA transferase was determined in mitochondria isolated from different rat hepatoma cell lines (Table 1). The activity range varied from 45 nmol/min/mg in H4-II-EC3 cells up to 960 nmol/min/mg in JM2 cells. The latter was about 200-fold greater than the trace amount of CoA transferase activity detected in normal rat hepatocytes. Observation of the doubling times of the various hepatoma cell lines indicated that there was an association between the cell growth rate and CoA transferase activity (Table 1). The activity of malate dehydrogenase was also determined and found to be approximately equivalent in all mitochondrial preparations (Table 1).

**Immunquantitation of CoA Transferase.** Equal quantities of mitochondrial protein from the different cell lines were subjected to SDS-PAGE analysis together with purified CoA transferase (Fig. 1A) and immunoblotted using anti-serum against the purified rat brain CoA transferase (Fig. 1B). The major band visualized in the rat hepatoma cell lines is slightly less than M, 55,000, which is the molecular weight of the rat brain enzyme (Fig. 1, lane 2). CoA transferase in normal hepatocyte mitochondria was not detectable by Western blotting (Fig. 1, lane 1). In the cell lines that have the least activity, RTL-3C, McA-RH7777, and H4-II-EC3 (Fig. 1, lanes 8–10) there is an immunodetectable band of slightly higher molecular weight than the major band. We do not know if this higher molecular weight band is a precursor of the lower molecular weight species. It should be noted that the cell line H4-II-EC3, which has the lowest CoA-transferase activity, expresses only the high-molecular-weight band. For purposes of quantification, all immunoreactive bands on the Western blots were scanned to determine relative levels of CoA transferase. These data are given in Table 1.

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**In Vivo Synthesis and in Vitro Translation.** To study further the basis for the increase in the CoA transferase activity in hepatoma cell lines, the biosynthesis of the enzyme was determined in perfusion-isolated normal hepatocytes and 3 hepatoma cell lines by using L-[35S]methionine incorporation followed by immunoprecipitation (Fig. 2). The synthesized en-

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**Table 1 CoA transferase activity and cell growth rate**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CoA transferase (nmol/min/mg)</th>
<th>Cell culture doubling time (h)</th>
<th>MDH activity (μmol/min/mg)</th>
<th>CoA transferase relative content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte</td>
<td>4.6 ± 2.1</td>
<td>ND</td>
<td>2.14 ± 0.13</td>
<td>ND</td>
</tr>
<tr>
<td>H4-II-EC3</td>
<td>45 ± 14</td>
<td>51</td>
<td>1.85 ± 0.25</td>
<td>19</td>
</tr>
<tr>
<td>McA-RH7777</td>
<td>194 ± 56</td>
<td>49</td>
<td>1.92 ± 0.16</td>
<td>48</td>
</tr>
<tr>
<td>RTL-3C</td>
<td>362 ± 93</td>
<td>32</td>
<td>2.02 ± 0.17</td>
<td>65</td>
</tr>
<tr>
<td>RTL-9F</td>
<td>495 ± 109</td>
<td>33</td>
<td>2.21 ± 0.21</td>
<td>70</td>
</tr>
<tr>
<td>RTL-2M</td>
<td>507 ± 113</td>
<td>20</td>
<td>2.89 ± 0.28</td>
<td>76</td>
</tr>
<tr>
<td>HCT</td>
<td>624 ± 140</td>
<td>21</td>
<td>2.31 ± 0.24</td>
<td>86</td>
</tr>
<tr>
<td>RTL-5G</td>
<td>887 ± 135</td>
<td>22</td>
<td>2.27 ± 0.22</td>
<td>100</td>
</tr>
<tr>
<td>JM2</td>
<td>960 ± 190</td>
<td>18</td>
<td>2.69 ± 0.19</td>
<td>100</td>
</tr>
</tbody>
</table>

* Mean ± SD, n = 3.
* MDH, malate dehydrogenase; ND, not determined.
Fig. 1. Mitochondrial protein SDS-PAGE and immunoblotting of CoA transferase. Equal amounts (100 μg) of total mitochondrial protein from each cell line were loaded and fractionated on 12% separating gels followed by Coomassie blue staining (A). The order of the samples from lanes 1–11 are molecular weight markers, purified brain CoA transferase, JM-2, RLT-56, HTC, RLT-2M, RLT-9F, RLT-3C, Mca-RH7777, H4-II-EC3, and normal hepatocytes. Antiserum against rat brain CoA transferase was used to detect the enzyme of rat hepatoma cells (B). The order of the samples is the same as for (A).

...zyme had a subunit size that was close to the size of the purified rat brain enzyme on SDS-PAGE (Fig. 2, lane 7). The location of CoA transferase was confirmed by using excess purified enzyme to block the immunoprecipitation, resulting in the disappearance of the enzyme band (Fig. 2, lane 6). Pre-immune blotting was used to reduce the contaminating bands (Fig. 2, lane 1). CoA transferase synthesis was not detectable in hepatocyte lysates (Fig. 2, lane 2). Increasing amounts of enzyme synthesis occurred in H4-II-EC3, RLT-3C, and JM2 cells as shown in Fig. 2, lanes 3–5. The relative quantitation of the synthesized enzyme was determined by scanning the autoradiogram (Table 2). Again, there was a good association between these results and the enzyme activity determinations.

The results of the in vivo synthesis are supported by determining the functional mRNA level of CoA transferase in total cellular RNA by in vitro translations. The translated CoA transferase was isolated by immunoprecipitation followed by SDS-PAGE and visualized by fluorography (Fig. 3). The results were quite similar to that of the in vivo synthesis except that the in vitro-synthesized CoA transferase was detected as a precursor, with an apparent molecular weight of about 65,000. The identity of the pre-CoA transferase was again determined by using an excess amount of the purified enzyme to block the immunoprecipitation of the precursor band (Fig. 3, lane 5). The relative quantitation of the translated pre-CoA transferase in different cell lines is also shown in Table 2.

**DISCUSSION**

Succinyl-CoA:acetoacetyl-CoA transferase normally exists at high levels in many tissues, a notable exception being the liver.
system for 120 min at 30°C. Total translation products were adjusted to equal
Total cellular RNAs (200 µg/ml) from rat hepatocytes and hepatoma cell lines
CoA transferase (30 µg). Lane 6, the purified CoA transferase as a standard. Lane
of normal hepatocytes. H4-11-EC3, RLT-3C, and JM2 cells. Lane 5, the blockage
transferase (preCoAT) immunoprecipitated from the translation products in the order
visualized by fluorography. Lanes 1-4 show the M, 65,000 precursor CoA trans-
amined resulted from an increase in the amount of CoA transferase protein in the cells. Furthermore, the results from in vivo biosynthesis studies together with in vitro translations indicate that the differences in enzyme level are the result of different levels of functional CoA-transferase mRNA.

However, the enzyme is expressed to varying amounts in hepatoma cell lines (14, 15). Our results indicate that the differential CoA transferase activities in the hepatoma cell line examined resulted from an increase in the amount of CoA transferase protein in the cells. Furthermore, the results from in vivo biosynthesis studies together with in vitro translations indicate that the differences in enzyme level are the result of different levels of functional CoA-transferase mRNA.

Regulation of CoA transferase has been demonstrated to occur not only in hepatoma cell lines but also in a variety of tissues and cells under different conditions. CoA transferase is differentially expressed in different rat tissues (5, 7, 8) and has been attributed to different amounts of CoA transferase mRNA existing in these tissues (9). It has been further reported that CoA transferase can be inducibly regulated within different subtypes of the same tissue, such as rat muscles (11). CoA transferase has also been shown to be subjected to either down-regulation in the heart of hyperthyroid rats (13) or up-regulation in proliferating cells, such as neonatal or regenerating rat hepatocytes (14), and perinatal rat brain cells (10, 12).

Rat brain CoA transferase is a mitochondrial protein composed of 2 identical subunits with a subunit M, 55,000 (24). In the present study, functional CoA transferase in mitochondria from rat hepatoma cells has a slightly smaller subunit size than the purified functional brain enzyme as detected by Western blots. However, the precursor CoA transferase synthesized for hepatoma cellular RNA during in vitro translations was approximately M, 65,000. Thus, the newly synthesized pre-CoA transferase has greater than M, 10,000 difference in subunit size from the mitochondrial mature enzyme. This is consistent with the reports that most mitochondrial proteins are synthesized as precursor polypeptides containing an NH2-terminal extension (signal peptide) with a molecular weight of approximately 2,000–10,000 (35, 36).

Ketone bodies have been shown to be important energy substrates in place of glucose in nonhepatic tissues (3, 10). CoA transferase is the enzyme that makes it possible for a given cell to utilize ketone bodies (4–7). The present results reveal that there is a relationship between CoA transferase expression in hepatoma cells and their growth rate. This suggests that CoA transferase may play a role in energy metabolism in hepatoma cells. Further experiments are needed to elucidate the mechanisms underlying the differential regulation of CoA transferase expression in these cells as well as the metabolic importance of the expression of the enzyme associated with the growth rate of hepatoma cells.

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REFERENCES

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