Malonyl-CoA-independent Acute Control of Hepatic Carnitine Palmitoyltransferase I Activity

ROLE OF Ca++/CALMODULIN-DEPENDENT PROTEIN KINASE II AND CYTOSKELETAL COMPONENTS

(Received for publication, February 18, 1998, and in revised form, May 29, 1998)

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The mechanism of malonyl-CoA-independent acute control of hepatic carnitine palmitoyltransferase I (CPT-I) activity was investigated. In a first series of experiments, the possible involvement of the cytoskeleton in the control of CPT-I activity was studied. The results of these investigations can be summarized as follows. (i) Very mild treatment of permeabilized hepatocytes with trypsin produced around 50% stimulation of CPT-I activity. This effect was absent in cells that had been pretreated with okadaic acid (OA) and seemed to be due to the action of trypsin on cell component(s) distinct from CPT-I. (ii) Incubation of intact hepatocytes with 3,3′-iminodipropionitrile, a disruptor of intermediate filaments, increased CPT-I activity in a non-additive manner with respect to OA. Taxol, a stabilizer of the cytoskeleton, prevented the OA- and 3,3′-iminodipropionitrile-induced stimulation of CPT-I. (iii) CPT-I activity in isolated mitochondria was depressed in a dose-dependent fashion by the addition of a total cytoskeleton fraction and a cytokeratin-enriched cytoskeletal fraction, the latter being 3 times more potent than the former. In a second series of experiments, the possible link between Ca++/calmodulin-dependent protein kinase II (Ca++/CM-PKII) and the cytoskeleton was studied in the context of CPT-I regulation. The data of these experiments indicate that (i) purified Ca++/CM-PKII activated CPT-I in permeabilized hepatocytes but not in isolated mitochondria, (ii) purified Ca++/CM-PKII abrogated the inhibition of CPT-I of isolated mitochondria induced by a cytokeratin-enriched fraction, and (iii) the Ca++/CM-PKII inhibitor KN-62 prevented the OA-induced phosphorylation of cytokeratins in intact hepatocytes. Results thus support a novel mechanism of short-term control of hepatic CPT-I activity which may rely on the cascade Ca++/CM-PKII activation → cytokeratin phosphorylation → CPT-I de-inhibition.

Mitochondrial fatty acid oxidation in liver provides a major source of energy to this organ and supplies extrahepatic tissues with ketone bodies as a glucose-replacing fuel (1, 2). Carnitine palmitoyltransferase I (CPT-I),3 the carnitine palmitoyltransferase of the mitochondrial outer membrane, catalyzes the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix (1–5). Moreover, recent determination of flux control coefficients of the enzymes involved in hepatic long-chain fatty acid oxidation shows that CPT-I plays a pivotal role in controlling the flux through this pathway under different substrate concentrations and pathophysiological states (6, 7). CPT-I is subject to long-term regulation in response to alterations in the nutritional and hormonal status of the animal (1, 2, 5). Short-term control of CPT-I activity involves inhibition by malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase (8). Since the latter enzyme is a key regulatory site of fatty acid synthesis de novo (cf. Refs. 1–5), malonyl-CoA inhibition of CPT-I allows an elegant explanation for the coordinate control of the partition of hepatic fatty acids into esterification and oxidation. As a matter of fact, evidence has accumulated during the last two decades highlighting the physiological importance of malonyl-CoA inhibition of CPT-I not only in liver but also in extrahepatic tissues (1, 5).

During the last years, however, a novel mechanism of control of hepatic CPT-I activity has been put forward. Studies using permeabilized hepatocytes have shown that various agents exert short-term changes in CPT-I activity in parallel with changes in the rate of long-chain fatty acid oxidation (3, 9). These short-term changes in hepatic CPT-I activity are assumed to be mediated by a malonyl-CoA-independent mechanism, since they survive cell permeabilization, extensive washing of the permeabilized cells (to allow complete removal of malonyl-CoA), and subsequent preincubation of the cell ghosts at 37 °C before determination of CPT-I activity (to allow recovery of the original conformational state of CPT-I) (10). Evidence has also been presented showing that the stimulation of hepatic CPT-I by the phosphatase inhibitor okadaic acid (OA), used as a model compound to study the short-term regulation of CPT-I, does not involve the direct phosphorylation of CPT-I (10). It has been recently shown that the OA-induced stimulation of CPT-I is prevented by KN-62, an inhibitor of Ca++/calmodulin-dependent protein kinase II (Ca++/CM-PKII) (11), and by taxol, a stabilizer of the cytoskeleton (12). These observations suggest that both activation of Ca++/CM-PKII and disruption of the cytoskeleton may be necessary for the OA-induced stimulation of CPT-I to be demonstrated. It is

3 The abbreviations used are: CPT-I, carnitine palmitoyltransferase I; Ca++/CM-PKII, Ca++/calmodulin-dependent protein kinase II; IDPN, 3,3′-iminodipropionitrile; OA, okadaic acid; PAGE, polyacrylamide gel electrophoresis; Pipes, 4-piperazinediethanesulfonic acid.
conceivable that these two processes may be related, since Ca\(^{2+}\)/CM-PKII is one of the protein kinases more actively involved in the control of the integrity of the cytoskeleton by phosphorylating cytoskeletal proteins (13). However, the events underlying this novel mechanism of control of CPT-I activity are as yet unknown. The present work was thus undertaken to study the molecular basis of the malonyl-CoA-independent short-term control of hepatic CPT-I activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—\(1\)-ethylin-\(^{3}H\)Carnitine, carrier-free \[^{32}P\]Pi, \[^{1-14}C\]acetyl-CoA, the ECL detection kit, and the protein kinase C assay kit were from Amersham International (Amersham, Bucks, United Kingdom). Tetracycloliglycide was kindly donated by Dr. J. M. Lowenstein (Brandeis University, Waltham, MA). The anti-CPT-I polyclonal antibody (raised against peptide residues 428–441 of rat liver CPT-I) was kindly given by Dr. V. A. Zammit (Hannah Research Institute, Ayr, United Kingdom). The anti-cytokeratin 8, anti-cytokeratin 18, and anti-actin monoclonal antibodies were kindly given by Dr. P. M. P. Bergen and Henegouwen (Utrecht University, The Netherlands). Colchicine and cytochalasin B were kindly gifted by Dr. J. M. Andreu (CIB, Madrid, Spain). 3,3’-Iminodipropionitrile (IDPN) was from Acrors Chimica (Geel, Belgium). OA, KN-62, A23187, and classical protein kinase C were from Sigma.

**cAMP-dependent Protein kinase, trypsin, and the anti-actin and anti-pan cytokeratin monoclonal antibodies were from ICN Pharmaceuticals (Costa Mesa, CA).** cAMP-dependent protein kinase, trypsin, and the anti-actin and anti-pan cytokeratin monoclonal antibodies were from Sigma.

**Isolation and Incubation of Hepatocytes—**Male Wistar rats (200–250 g) which had free access to food and water were used in all experiments. Hepatocytes were isolated by the collagenase perfusion method and routinely incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 10 mM glucose and 1% (w/v) defatted and dialyzed bovine serum albumin. Hepatocytes (6–8 mg of cellular protein in 1.5 ml of the aforementioned medium) were subsequently incubated in that medium at 37 °C for 1 h with 0.2 mCi of \[^{32}P\]Pi, and subsequently exposed to the additions indicated. One ml of cells was rapidly sedimented (5 s at 12,000 \(\times g\)) and resuspended in 0.5 ml of 50 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% (w/v) Igepal, 0.5% (w/v) sodium deoxycholate, 5 mM Tris-\(\cdot\)HCl, and 0.1% (w/v) SDS, supplemented with proteinase inhibitors (11, 15). Further treatment of the samples and immunoprecipitation with the monoclonal anti-pan cytokeratin antibody bound to protein A-Sepharose were performed as described (11, 15).

**Western Blot Analysis of Cytoskeletal Proteins—**Cytoskeletal fractions were subjected to SDS-PAGE using 10% polyacrylamide gels and proteins were further transferred onto nitrocellulose membranes. The blots were then blocked with 2% Protifar (Nutricia, Zoetermeer, The Netherlands) in 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, and 0.1% Tween 20 (TBST). They were subsequently incubated with the anti-actin, anti-tubulin, anti-cytokeratin 8, and anti-cytokeratin 18 monoclonal antibodies (11,10) in TBST with 0.2% Protifar for 1 h at 4 °C. The blots were then treated with polyethylene glycol for 1 h at room temperature, and finally subjected to luminography with the ECL detection kit.

**Immunoprecipitation of \[^{32}P\]-Labeled Cytokeratins—**After isolation, hepatocytes were washed twice in phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 1% (w/v) defatted and dialyzed bovine serum albumin. Hepatocytes (6–8 mg of cellular protein in 1.5 ml of the aforementioned medium) were subsequently incubated in that medium at 37 °C for 1 h with 0.2 mCi of \[^{32}P\]Pi, and subsequently exposed to the additions indicated. One ml of cells was rapidly sedimented (5 s at 12,000 \(\times g\)) and resuspended in 0.5 ml of 50 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% (w/v) Igepal, 0.5% (w/v) sodium deoxycholate, 5 mM Tris-\(\cdot\)HCl, and 0.1% (w/v) SDS, supplemented with proteinase inhibitors (11, 15). Further treatment of the samples and immunoprecipitation with the monoclonal anti-pan cytokeratin antibody bound to protein A-Sepharose were performed as described (11, 15).

**Determination of the Stoichiometry of Cytokeratin Phosphorylation—**The stoichiometry of cytokeratin phosphorylation was calculated by simultaneously determining (i) the specific radioactivity of the γ-phosphate of intracellular ATP, (ii) the amount of \[^{32}P\] incorporated into the cytokeratin bands, and (iii) the mass of protein in those cytokeratin bands.

(i) To determine the specific radioactivity of the γ-phosphate of intracellular ATP, hepatocytes were labeled with \[^{32}P\]Pi for 60 min to achieve steady-state labeling of proteins before addition of the agonists (16). After the indicated times, 1.0 ml of cells was precipitated with 0.15 ml of 2 × HClO\(_4\). After neutralization with K\(_2\)CO\(_3\), samples were centrifuged (20,000 \(\times g\), 15 min). Supernatants were filtered through a filter of 0.22-μm pore diameter and subsequently used for nucleotide separation exactly as described by Gualix et al. (17). The specific radioactivity of nucleotides was determined by measuring in parallel the nucleotide concentration after transformation of the A\(_{259}\) peak areas to the corresponding cAMP phosphorylation (17). The specific radioactivity of nucleotides was determined by measuring in parallel the nucleotide concentration after transformation of the A\(_{259}\) peak areas to the corresponding cAMP phosphorylation (17). The specific activity of the γ-phosphate of ATP was taken to be the difference between the specific activities of ATP and ADP (18, 19).

(ii) To determine the amount of \[^{32}P\] incorporated into cytokeratins, immunoprecipitates were obtained and treated exactly as described above, the two labeled bands in the gels were cut out and their radioactivity was determined.

(iii) To determine the amount of protein in the labeled bands corresponding to cytokeratins 8 and 18, immunoprecipitates were obtained and treated exactly as described above except that \[^{32}P\]Pi was omitted from the hepatocyte incubation medium. Immunoprecipitates were subjected to SDS-PAGE together with varying concentrations of purified cytokeratins 8 and 18. Band areas were visualized by the silver staining technique (20), and the amount of protein in the cytokeratin bands was calculated by interpolating the values of optical density of the immunoprecipitated protein bands in the standard curve of protein mass versus optical density constructed with the commercial, purified cyto- keratins 8 and 18. The standard curve was corrected for the contaminating proteins present in the commercial preparations of purified cytokeratins 8 and 18. In order to improve the visualization of the stained protein bands of the immunoprecipitate (e.g. Fig. 6), the amount of immunoprecipitate applied for determination of cytokeratin mass was routinely 3 times the amount used for other purposes (e.g. cyto- keratin phosphorylation, Western blotting). Values of cytokeratin mass inferred were corrected for this factor.

**Isolation of Cytoskeletal Fractions—**Isolated hepatocytes were sedimented (2 min at 100 \(\times g\)) and resuspended in a cytoskeleton stabilizing buffer consisting of 10 mM Pipes (pH 6.8), 0.25 m Na succrose, 3 mM MgCl\(_2\), 150 mM KC1, and 1 mM EGTA, supplemented with a proteinase/ inhibitor mixture (11, 15). The fraction corresponding to total cytoskeleton (Fraction I) as well as the fraction enriched in intermediate filaments (Fraction II) were prepared according to van Bergen and Henegouwen et al. (14).

**Western Blot Analysis of Cytoskeletal Proteins—**Cytoskeletal fractions were subjected to SDS-PAGE using 10% polyacrylamide gels and proteins were further transferred onto nitrocellulose membranes. The blots were then blocked with 2% Protifar (Nutricia, Zoes- termeer, The Netherlands) in 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, and 0.1% Tween 20 (TBST). They were subsequently incubated with the anti-actin, anti-tubulin, anti-cytokeratin 8, and anti-cytokeratin 18 monoclonal antibodies (11,10) in TBST with 0.2% Protifar for 1 h at 4 °C. The blots were then treated with polyethylene glycol for 1 h at room temperature, and finally subjected to luminography with the ECL detection kit.
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Effect of mild trypsin digestion on CPT-I activity in permeabilized hepatocytes. Hepatocytes were preincubated for 15 min in the absence (○) or presence (●) of 0.5 μM OA. Cells were then permeabilized with digitonin and thoroughly washed with 40 volumes of digitonin-free medium as described under “Experimental Procedures.” Permeabilized hepatocytes were subsequently resuspended in 1.5–2.0 mg of protein/ml and treated with varying concentrations of trypsin at 4 °C. Trypsin action was stopped after 2 min by addition of 10 mg/ml bovine serum albumin and immediate washing with 40 volumes of trypsin-free medium. CPT-I activity was subsequently determined in those permeabilized hepatocytes. One-hundred percent CPT-I activity was 1.87 ± 0.20 nmol/min × mg of protein. Results correspond to four different cell preparations. Inset, mitochondria were isolated from permeabilized hepatocytes that had been treated without (lane a) or with (lane b) 17.5 μg/ml trypsin, and CPT-I was detected by Western blotting. The arrow points to the 88-kDa band.

To test whether trypsin may cleave CPT-I itself under these digestion conditions, mitochondria were isolated from control and trypsin-treated permeabilized hepatocytes and CPT-I was subsequently detected by Western blotting (Fig. 1). As expected (5), a major band with a molecular mass of 88 kDa was detected in the blots. In addition, no differences were observed between the two preparations of mitochondria (Fig. 1). Thus, the value of relative optical density of the 88-kDa band of trypsin-treated samples was 100 ± 4% (n = 4), setting at 100% the value for control mitochondria. Furthermore, CPT-I activity was determined in mitochondria isolated from permeabilized hepatocytes that had been treated with or without trypsin. As shown in Table I, no differences in CPT-I activity were evident among the different conditions.

It is worth noting that treatment of permeabilized hepatocytes with trypsin had no effect on the recovery of total permeabilized cell or total mitochondrial protein. Thus, when permeabilized hepatocytes at 1.6 ± 0.2 mg of protein (n = 4) were treated without or with 17.5 μg of trypsin for 2 min at 4 °C and ghosts were collected after stopping trypsin action as described in legend to Fig. 1, 1.5 ± 0.1 and 1.5 ± 0.2 mg of ghost protein were recovered, respectively. Likewise, when mitochondria were isolated from those ghosts, 0.18 ± 0.04 and 0.19 ± 0.06 mg of protein were recovered in mitochondria prepared from trypsin-treated and trypsin-untreated ghosts, respectively.

Since permeabilized hepatocytes also express CPT activity from peroxisomes and microsomes (cf. Refs. 1–5), the contribution of CPT-I to total hepatocellular tetradeceylglycinate-sensitive CPT activity was quantified. Thus, hepatocytes were incubated with 10 μM tetradeceylglycinate for 30 min; purified mitochondria, peroxisomes, and microsomes were isolated (9), and CPT activity was measured in these fractions. It turned out that at least 85% of total tetradeceylglycinate-sensitive CPT activity experimentally determined routinely corresponded to CPT-I, whereas microsomal CPT and peroxisomal CPT together made a minor contribution (<15%) to the tetradeceylglycinate-sensitive CPT pool under these conditions. Therefore, we believe that determination of CPT-I activity by our permeabilized hepatocyte procedure is not prone to a substantial error.

| Hepatocyte preincubation | Trypsin |
|--------------------------|--------|
|                         | Permeabilized hepatocytes | Isolated mitochondria |
| No additions             | 2.04 ± 0.21 | 7.88 ± 0.93 |
| Yes                     | 3.01 ± 0.13* | 7.81 ± 0.67 |
| Taxol                    | 1.98 ± 0.34 | 8.05 ± 1.33 |
| Yes                     | 2.87 ± 0.16* | 7.94 ± 0.41 |
|OA                       | 3.00 ± 0.47* | 8.02 ± 0.72 |
|Yes                      | 3.12 ± 0.58* | 7.32 ± 0.50 |
| Taxol + OA               | 2.11 ± 0.30 | 7.57 ± 0.88 |
|Yes                      | 2.98 ± 0.49* | 8.07 ± 1.05 |

*Significantly different (p < 0.01) from incubations with no additions.
**Effect of disruptors of the cytoskeleton on CPT-I activity and malonyl-CoA levels**

Hepatocytes were preincubated for 45 min in the absence or presence of the modulators of the integrity of the cytoskeleton, as indicated. Incubations were continued for 15 min additional with or without 0.5 μM OA, and then aliquots were taken to determine the level of malonyl-CoA as well as the activity of CPT-I by the one-step assay. One-hundred percent values of CPT-I activity and malonyl-CoA concentration were 1.29 ± 0.22 pmol/min × mg of protein and 73 ± 12 pmol/mg of protein, respectively. Results correspond to the number of experiments indicated in parentheses for CPT-I activity and to three different experiments for malonyl-CoA concentration.

| Hepatocyte preincubation | OA | CPT-I activity | Malonyl-CoA |
|--------------------------|----|----------------|-------------|
| No additions             | No | 100 ± 17 (8)   | 100 ± 16    |
|                          | Yes| 154 ± 15 (8)   | 7 ± 5*      |
| Colchicine (50 μM)       | No | 98 ± 12 (4)    | 96 ± 14     |
|                          | Yes| 155 ± 11* (4)  | 5 ± 6*      |
| Cytochalasin B (10 μM)   | No | 96 ± 11 (4)    | 104 ± 21    |
|                          | Yes| 151 ± 18* (4)  | 7 ± 3*      |
| IDPN (5 mM)              | No | 143 ± 11* (6)  | 103 ± 15    |
|                          | Yes| 166 ± 15* (6)  | 6 ± 6*      |
| Taxol (10 μM)            | No | 102 ± 9 (4)    | 101 ± 12    |
|                          | Yes| 104 ± 8 (4)    | 13 ± 8*     |
| Taxol (10 μM) + IDPN (5 mM) | No | 96 ± 6 (6)     | 13 ± 9      |
|                          | Yes| 99 ± 5 (6)     | 7 ± 4*      |

*Significantly different (p < 0.01) from incubations with no additions.

**Effect of Cytoskeletal Fractions on CPT-I Activity**—In order to define the cell components that are sufficient for the malonyl-CoA-independent control of CPT-I to be demonstrated, we next attempted to reconstitute the whole-cell experimental system in a simple manner by incubating isolated mitochondria together with cytoskeletal Fraction II and purified Ca\(^{2+}\)/CM-PKII. As shown in Fig. 4, the inhibition of CPT-I produced by exposure of isolated mitochondria to cytoskeletal Fraction II was reverted by addition of exogenous Ca\(^{2+}\)/CM-PKII.

**Phosphorylation of Cytokeratins in Intact Hepatocytes**—To obtain further evidence for a possible connection between Ca\(^{2+}\)/CM-PKII and intermediate filaments, hepatic cytoskeleton phosphorylation was investigated. The phosphorylation pattern of purified cytokeratins in situ may not reflect their phosphorylation status in more physiological, intact cell systems (27–29). Therefore, intact hepatocytes labeled with \(^{32}\)P, and cytokeratins were immunoprecipitated. As shown in Fig. 5, two major cytokeratin bands were phosphorylated upon hepatocyte challenge to OA. These two bands were assigned to cytokeratins 8 and 18 on the basis of their molecular mass (54 and 45 kDa, respectively) and high abundance in rat liver (e.g. Refs. 27–29). Moreover, the OA-induced phosphorylation of these two bands was prevented by KN-62, the Ca\(^{2+}\)/CM-PKII
or not (2) correspond to four different experiments. *, significantly different (p < 0.01) from incubations with no additions.

Aliquots were subsequently taken to determine CPT-I activity. Results of the immunoprecipitated proteins was performed with the anti-cytokeratin 8 and anti-cytokeratin 18 monoclonal antibodies. The rationale of this experiment was that it would be most unlikely that proteins different to cytokeratins would also cross-react with the anti-cytokeratin antibodies on a Western blot, especially when, as in the present study, different sources of antibodies are used in the immunoprecipitation and in the blotting. As shown in Fig. 6, the 54-kDa band actually contained cytokeratin 8, whereas cytokeratin 18 was actually present in the 45-kDa band.

Stoichiometry of Cytokeratin Phosphorylation—Since cytokeratins are rather abundant elements of the hepatocyte cytoskeleton (27–29), it might be argued that the stoichiometry of cytokeratin phosphorylation might be very low and therefore non-functional. Therefore, the phosphorylation state of cytokeratins in okadaic acid-treated hepatocytes was determined. For this purpose, we determined the specific radioactivity of ATP, ADP, AMP in the phosphorylated proteins reaches isotopic equilibrium with the γ-phosphate of intracellular ATP by a high performance liquid chromatography method, the amount of 32P incorporated into the cytokeratins, and the amount of cytokeratins in the immunoprecipitated bands as described under "Experimental Procedures." This calculation assumes that all of the phosphate in the phosphorylated proteins reaches isotopic equilibrium with the γ-phosphate of intracellular ATP after the 60-min labeling period with 32Pi, (16, 18). After this 60-min labeling period, the ratio of specific radioactivities of ATP:ADP:AMP was 1.00:0.62:0.15 and 1.00:0.56:0.11 (Table III). These values are in agreement with those obtained by Holland et al. and indicate that the β- and the γ-phosphates are at isotopic equilibrium with each other, but not with the α-phosphate. Fig. 6 shows a gel used for the calculation of the amount of protein in the 54- and 45-kDa bands. Values of mole of phosphate/mole of cytokeratin obtained in two separate experiments (Table III) are in agreement with the observations that cytokeratins 8 and 18 (and mostly the former) become significantly phosphorylated upon exposure of intact hepatocytes to phosphatase inhibitors (22, 28), although in the latter two reports no quantification of cytokeratin phosphorylation was achieved. Therefore, cytokeratin phosphorylation may be functionally relevant in our system.

DISCUSSION

Involvement of Cytoskeletal Components in the Control of CPT-I Activity—A number of reports have recently described the existence of specific interactions between the mitochondrial outer membrane and cytoskeletal elements (reviewed in Refs. 30 and 31). Four observations in the present report strengthen the notion that cytoskeletal components (most likely cytokeratin intermediate filaments) are involved in the control of hepatic CPT-I activity. First, experiments of mild trypsin diges-
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Fig. 6. Quantification of the amount of cytokeratins 8 and 18 in the immunoprecipitate obtained with the anti-pan cytokeratin monoclonal antibody. Panel A, the immunoprecipitate obtained with the anti-pan-cytokeratin monoclonal antibody was resolved by SDS-PAGE, blotted, incubated with anti-rat-cytokeratin 8 monoclonal antibody (lane a), or with anti-rat-cytokeratin 18 monoclonal antibody (lane b), and developed with an anti-rat peroxidase-conjugated secondary antibody. The arrows point to the 54-kDa band (ck8, lane a) or the 45-kDa band (ck18, lane b). Panel B, the immunoprecipitate obtained with the anti-pan cytokeratin monoclonal antibody (lane a) and varying amounts of commercial purified cytokeratin 8 (lane f, 10 ng; lane g, 20 ng; lane h, 40 ng; lane i, 60 ng) were resolved by SDS-PAGE and stained by the silver staining method. "ab" denotes the heavy chains of the anti-pan-cytokeratin antibody, which have a molecular mass of about 50 kDa.

| Parameter                              | Experiment 1 | Experiment 2 |
|----------------------------------------|--------------|--------------|
| Specific radioactivity of adenine      |              |              |
| nucleotides (dpm/mmol)                 |              |              |
| AMP                                    | 98           | 70           |
| ADP                                    | 415          | 341          |
| ATP                                    | 670          | 612          |
| ATP (γ-phosphate)                      | 255          | 271          |
| **CK8 phosphorylation**                |              |              |
| Picomole of phosphate in 54-kDa band   | 0.91         | 1.10         |
| Picomole of protein in 54-kDa band     | 0.16         | 0.18         |
| Picomole of phosphate/mole of CK8      | 5.7          | 6.1          |
| **CK18 phosphorylation**               |              |              |
| Picomole of phosphate in 45-kDa band   | 0.26         | 0.30         |
| Picomole of protein in 45-kDa band     | 0.07         | 0.09         |
| Mole of phosphate/mole of CK18         | 3.7          | 3.3          |

The possibility that CPT-I interacts with cytoskeletal components as put forward in this paper is in line with the current notion that the dynamics of mitochondria in living cells may result from specific interactions of mitochondria with components of the cytoskeleton. It has been suggested that a function of the interactions between mitochondria and intermediate filaments may be to locate mitochondria in precise sites within the cell. This idea is based on both in vitro (39, 40) and in vivo (41, 42) experiments. Since the organization of intermediate filaments changes dramatically in a number of liver pathologies (43), the observations described in the present paper predict that CPT-I activity as affected by cytoskeletal components may change under pathophysiological situations in which the organization of the cytoskeleton is altered, e.g., in transformed cells. In this respect, Paumen et al. (44) have recently observed that inhibition of CPT-I with etomoxir leads to a stimulation of ceramide synthesis and to palmitate-induced cell death. These authors suggested that cells that express high CPT-I activity are expected to withstand palmitate-induced apoptosis (44). Thus, we have recently observed that CPT-I specific activity is similar in mitochondria isolated from hepatoma cells and normal hepatocytes, but just about half in permeabilized hepatocytes than in permeabilized hepatoma cells; in addition, CPT-I is not activated by OA in hepatoma cells (45). These observations support the notion that in hepatocytes OA liberates CPT-I from certain constraints imposed by extramitochondrial cell components that do not operate either in isolated mitochondria or in transformed liver cells. Whether liberation of CPT-I from those potential constraints may help hepatoma cells to escape from apoptosis is currently under study in our laboratories. Anyway, it is worth noting that treatment of hepatocytes with OA, a well known tumor promoter, renders a “CPT-I regulatory phenotype” similar to that shown by hepatoma cells.

Involvement of Ca2+/CM-PKII in the Control of CPT-I Activity—Previous experiments in our laboratories have shown that KN-62, an inhibitor of Ca2+/CM-PKII (26), antagonizes the OA-induced stimulation of hepatic CPT-I activity (11). Data in the present report further point to a link between Ca2+/CM-PKII and the cytoskeleton in the context of CPT-I regulation. This conclusion is based mostly on three observations. First, CPT-I activity in isolated mitochondria was depressed in a dose-dependent fashion by the addition of a total cytosome fraction and a cytokeratin-enriched cytosome fraction, the latter being 3 times more potent than the former. Fourth, taxol prevented the OA-induced desensitization of CPT-I to trypsin activation, as well as the OA- and IDPN-induced stimulation of CPT-I. In short, all these data suggest that disruption of interactions between CPT-I and cytoskeletal component(s) may de-inhibit CPT-I and, therefore, increase enzyme activity.
simple reconstituted system composed of isolated mitochondria, a cytokeratin-enriched fraction, and purified Ca\textsuperscript{2+}/CaMKII may reflect the situation occurring in the intact hepatocyte, indicating that these three components are sufficient for the malonyl-CoA-independent acute control of CPT-I to be demonstrated in vitro. Third, the Ca\textsuperscript{2+}/CaMK-II inhibitor KN-62 prevented the OA-induced phosphorylation of cytokeratins in intact hepatocytes, pointing to a role of Ca\textsuperscript{2+}/CaMK-II on cytokeratin phosphorylation in these cells (28, 29).

Additional evidence for the involvement of cytokeratins in the control of CPT-I activity is given by the lack of effect of A23187 on cytokeratin phosphorylation in hepatocytes. In this context, challenge of hepatocytes to OA leads to CPT-I activation and cytokeratin phosphorylation, whereas elevation of cytosolic free Ca\textsuperscript{2+} concentration by A23187 has no effect on either CPT-I activity (47) or cytokeratin phosphorylation (the present paper). The possibility that liver Ca\textsuperscript{2+}/CaMK-II has a different pattern of activation by Ca\textsuperscript{2+}/calmodulin and by autophosphorylation than brain Ca\textsuperscript{2+}/CaMK-II (cf. Ref. 11) is as yet an open question.

It is worth noting that neither Ca\textsuperscript{2+}-dependent protein kinase nor protein kinase C affected CPT-I activity in permeabilized hepatocytes. This is in line with the observation that neither Ca\textsuperscript{2+}-dependent protein kinase inhibitors nor protein kinase C inhibitors were able to prevent the OA-induced stimulation of CPT-I (11). As a matter of fact, several reports indicate that, despite their ability to phosphorylate cytokertins in vitro, neither of these two protein kinases play an important role in the direct control of intermediate filament integrity in intact hepatocytes (28, 48, 49). In contrast, and in line with data in the present paper, Ca\textsuperscript{2+}/CaMK-II has been shown to play a major role in the phosphorylation and functional integrity of hepatic cytokeratins in vitro (28) as well as in the OA-induced disruption of hepatic cytoskeleton (21).

Malonyl-CoA-dependent and Malonyl-CoA-independent Control of CPT-I Activity—Together with previous observations (10–12), data in this paper allow for a model that explains the OA-induced malonyl-CoA-independent control of hepatic CPT-I. As shown in Scheme I, OA may activate Ca\textsuperscript{2+}/CaMK-II by increasing its degree of phosphorylation upon inhibition of protein phosphatases 1 and 2A; this effect would be overcome by KN-62, an inhibitor of Ca\textsuperscript{2+}/CaMK-II autophosphorylation. Activated Ca\textsuperscript{2+}/CaMK-II would phosphorylate cytoskeletal components, perhaps cytokeratins 8 and 18, thereby disrupting putative inhibitory interactions between the cytoskeleton and CPT-I. Stimulation of CPT-I upon disruption of the cytoskele-ton would be also achieved by challenge of intact hepatocytes to IDPN or by treatment of permeabilized hepatocytes with trypsin in mild conditions. Stabilization of the cytoskeleton with taxol may prevent the malonyl-CoA-independent acute stimulation of CPT-I.

It is obvious that the notion that fatty acid translocation into mitochondria may be controlled by modulation of the interactions between CPT-I and cytoskeletal components (i.e. by a malonyl-CoA-independent mechanism) does not diminish the importance of malonyl-CoA as a physiological modulator of CPT-I activity (5, 8). On the one hand, since the pioneering work of McGarry and co-workers (8, 50), changes in long-chain fatty acid oxidation under many different pathophysiological situations have been shown to be linked to changes in intracellular malonyl-CoA concentration and/or changes in the sensitivity of CPT-I to malonyl-CoA (1, 2, 5). On the other hand, several observations suggest that malonyl-CoA-dependent and malonyl-CoA-independent acute control of hepatic CPT-I activity might operate in concert. First, we have recently shown that stimulation of the AMP-activated protein kinase, a major protein kinase involved in the control of hepatic lipid metabolism, leads to an activation of hepatic CPT-I by malonyl-CoA-dependent and malonyl-CoA-independent mechanisms (51). Second, a fraction of hepatic acetyl-CoA carboxylase, the enzyme responsible for the synthesis of malonyl-CoA, has been recently suggested to be bound to the cytoskeleton (52). Third, it has been put forward that the 280-kDa isoform of acetyl-CoA carboxylase might interact with the outer leaflet of the mitochondrial outer membrane in order to channel malonyl-CoA for CPT-I inhibition (53). Fourth, the recent observation that the bulk of the CPT-I protein seems to face the cytoplasmic side of the mitochondrial outer membrane (34) makes more likely that interactions between CPT-I and cytoskeletal components might occur. Although the physiological role of the malonyl-CoA-independent mechanism of regulation of hepatic CPT-I activity is as yet unknown, it is worth noting that hormonal challenge of hepatocytes (e.g. glucagon, insulin) leads to changes in CPT-I activity that parallel changes in long-chain fatty acid oxidation and that are retained after washing of the permeabilized cells (3). In the context of the emerging role of cytoskeletal filamentous networks in intracellular signaling (54), current research in our laboratories is focused on the possible existence of a coordinate control of CPT-I and acetyl-CoA carboxylase activities by modulation of interactions between the cytoskeleton and the mitochondrial outer membrane.

Acknowledgments—We are indebted to Dr. Ismael Galve for expert technical assistance; Dr. Alejandro Alonso, Dr. Jean Francois Leterrier, and Dr. Paul M. P. Van Bergen en Henegouwen for advice in the isolation of cytoskeletal fractions; Dr. David Carling, Dr. Javier Guixal, and Dr. Antonio Sillero for advice in the phosphorylation experiments; and Dr. Lambert M.G. Van Golde for advice in preparing the manuscript.

REFERENCES
1. McGarry, J. D., Woelte, K. F., Kawajima, M., and Foster, D. W. (1989) Diabetes Metab. Res. 5, 271–284
2. Zammit, V. A. (1994) Diabetes Res. 2, 132–155
3. Guzmán, M., and Geelen, M. J. H. (1993) Biochim. Biophys. Acta 1167, 227–241
4. Eaton, S., Bartlett, K., and Pourfarzam, M. (1996) Biochem. J. 320, 345–357
5. McGarry, J. D., and Brown, N. F. (1997) Eur. J. Biochem. 244, 1–14
6. Drynan, L., Quant, P. A., and Zammit, V. A. (1996) Biochem. J. 317, 791–795
7. Spurr, T. D., Sherrat, H. S. A., Pogson, C. L., and Agius, L. (1997) Biochem. J. 323, 119–122
8. McGarry, J. D., and Foster, D. W. (1980) Annu. Rev. Biochem. 49, 385–420
9. Guzmán, M., and Geelen, M. J. H. (1992) Biochem. J. 287, 487–492
10. Guzmán, M., Kolodziejczyk, M. P., Caldwell, A., Costorphine, C. G., and Zammit, V. A. (1994) Biochem. J. 300, 683–699
11. Velasco, G., Guzmán, M., Zammit, V. A., and Geelen, M. J. H. (1997) Biochem. J. 321, 211–216
12. Velasco, G., Sánchez, C., Geelen, M. J. H., and Guzmán, M. (1996) Biochem. Biophys. Res. Commun. 224, 754–759
13. Braun, A. P., and Schulman, H. (1995) Annu. Rev. Physiol. 57, 417–445

![Scheme I. Proposed model for the malonyl-CoA-independent acute control of hepatic CPT-I activity. See the text for abbreviations and further details. +, activation; −, inhibition.](image-url)
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14. Van Bergen en Henegouwen, P. M. P., den Hartigh, J. C., Romeyn, P., Verkleij, A. J., and Boonstra, J. (1992) Exp. Cell Res. 199, 90–97

15. Guzmán, M., Bijleveld, C., and Geelen, M. J. H. (1995) Biochem. J. 311, 853–860

16. Avruch, J., Witters, L. A., Alexander, M. C., and Bush, M. A. (1978) J. Biol. Chem. 253, 4754–4761

17. Gualix, J., Abal, M., Pinter, J., García-Carmona, F., and Miras-Portugal, M. T. (1996) J. Biol. Chem. 271, 1957–1965

18. Netherlands, B., Witters, L. A., and Hardie, D. G. (1984) Eur. J. Biochem. 140, 325–333

19. Haystead, T. A. J., and Hardie, D. G. (1988) Eur. J. Biochem. 175, 339–345

20. Ohsawa, K., and Ebata, N. (1983) Anal. Biochem. 135, 409–415

21. Holen, I., Gordon, P. B., and Seglen, P. O. (1992) Biochem. J. 284, 633–636

22. Ohta, T., Nishiwaki, R., Yatsumami, J., Komori, A., Suganuma, M., and Fujiki, H. (1992) Carcinogenesis 13, 2443–2447

23. Feulloley, M., Contesse, V., Lefebvre, H., Delarue, C., and Vaudry, H. (1994) Am. J. Physiol. 266, E202–E210

24. De Loof, A., Broeck, J., and Janssen, I. (1996) Int. Rev. Cytol. 166, 1–58

25. Haagsman, H. P., de Haas, C. G. M., van Golde, L. M. G., and Geelen, M. J. H. (1981) Biochim. Biophys. Acta 665, 1–7

26. Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., and Hidaka, H. (1990) J. Biol. Chem. 265, 4315–4320

27. Fuchs, E., and Weber, K. (1994) Annu. Rev. Biochem. 63, 345–382

28. Tövola, D. M., Goldman, R. D., Garrod, D. K., and Eriksson, J. (1997) J. Cell Biol. 110, 23–33

29. Inagaki, M., Inagaki, N., Takahashi, T., and Takay, Y. (1997) J. Biochem. (Tokyo) 121, 407–414

30. Bereteiter-Hahn, J., and Vith, M. (1994) Microsc. Res. Tech. 27, 198–219

31. Leterrier, J. F., Rusakov, D. A., Nelson, B. D., and Lindeën, M. (1994) Microsc. Res. Tech. 27, 233–261

32. Fontaine, E. M., Keriel, C., Lantuejoul, S., Rigoulet, M., Levere, X. M., and Saks, S. A. (1995) Biochem. Biophys. Res. Commun. 213, 138–146

33. Kashfi, K., and Cook, G. A. (1992) Biochem. J. 282, 909–914

34. Epler, F., Costorphine, C. G., and Zammit, V. A. (1997) Biochem. J. 323, 711–718

35. Lindeën, M., and Karlsson, G. (1996) Biochem. Biophys. Res. Commun. 218, 833–836

36. Sterner, M. H., and Bedayan, M. (1990) Cell Motil. Cytoskeleton 17, 11–18

37. Almabiliobi, G., Williams, L. J., and Hall, P. F. (1992) Exp. Cell Res. 200, 361–369

38. Bernier-Valentin, F., and Rousett, B. (1982) J. Biol. Chem. 257, 7092–7099

39. Eckert, L. S. (1986) Cell Motil. Cytoskeleton 6, 15–24

40. Collier, N. C., Sheetz, M. P., and Schlesinger, M. J. (1993) J. Cell. Biochem. 52, 297–397

41. Li, Z., Coluci-Guyon, E., Pincon-Raymon, M., Merickay, M., Pouinin, S., Paulio, D., and Babinet, C. (1996) Dev. Biol. 175, 362–366

42. Milner, D. J., Weitzer, G., Tran, D., Bradley, A., and Capetanaki, Y. (1996) J. Cell Biol. 134, 1255–1270

43. Omary, M. B., and Ku, N. O. (1997) Hepatology 25, 1043–1048

44. Paumen, M. P., Isida, Y., Muramatsu, M., Yamamoto, M., and Honjo, T. (1997) J. Biol. Chem. 272, 3324–3329

45. Velasco, G., Passilly, P., Guzmán, M., and Latruffe, N. (1996) Biochem. Pharmacol., in press

46. Fiskum, M., Craig, S. W., Becker, G. L., and Lehnagew, A. L. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3430–3434

47. Guzmán, M., Velasco, G., and Castro, J. (1996) Am. J. Physiol. 270, G707–G707

48. Ku, N. O., and Omary, B. (1994) J. Cell Biol. 127, 161–171

49. Cadrin, M., McFarlane-Anderson, N., Aasheem, L. H., Kawahara, H., Franks, D. J., Marceau, N., and French, S. W. (1992) Cell. Signalling 4, 715–722

50. McGorry, C. J., Mannaeurs, G. F., and Foster, D. W. (1977) J. Clin. Invest. 60, 265–270

51. Velasco, G., Geelen, M. J. H., and Guzmán, M. (1997) Arch. Biochem. Biophys. 337, 169–175

52. Geelen, M. J. H., Bijleveld, C., Velasco, G., Wandes, R. A. J., and Guzmán, M. (1997) Biochem. Biophys. Res. Commun. 233, 253–257

53. Ha, J., Lee, J. K., Kim, S. W., Witters, L. A., and Kim, K. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 83, 11466–11470

54. Forges, G. (1995) J. Cell Sci. 108, 2131–2143