Expression of novel isoforms of carnitine palmitoyltransferase I (CPT-1) generated by alternative splicing of the CPT-Iβ gene

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Carnitine palmitoyltransferase I (CPT-I) catalyses the rate-determining step in mitochondrial fatty acid β-oxidation. The enzyme has two cognate structural genes that are preferentially expressed in liver (α) or fat and muscle (β). We hypothesized the existence of additional isoforms in heart to account for unique kinetic characteristics of enzyme activity in this tissue. Hybridization and PCR screening of a human cardiac cDNA library revealed the expression of two novel CPT-I isoforms generated by alternative splicing of the CPT-Iβ transcript, in addition to the β and α cDNA species previously described. Ribonuclease protection and reverse transcriptase-mediated PCR assays confirmed the presence of mRNA species of each splicing variant in heart, skeletal muscle and liver, with differing relative concentrations in the tissues. The novel splicing variants omit exons or utilize a cryptic splice donor site within an exon. Deduced polypeptide sequences of the novel enzymes include omissions in the region of putative membrane-spanning and malonyl-CoA regulatory domains compared with the previously described CPT-Is, implying that the encoded enzymes will exhibit unique features with respect to outer mitochondrial membrane topology and response to physiological and pharmacological inhibitors.

INTRODUCTION

The carnitine shuttle is utilized in mammalian cells for the entry of long-chain fatty acids into the mitochondrial matrix, where they undergo β-oxidation [1,2]. Carnitine palmitoyltransferase I (EC 2.3.1.21; CPT-I) spans the outer mitochondrial membrane and catalyses the transfer of fatty acyl groups from CoA to carnitine. Acylcarnitines thus formed traverse the inner membrane via a specific translocase, whereupon fatty acyl-CoAs are regenerated by CPT-II within the matrix. This shuttle constitutes the rate-determining process in fatty acid oxidation in all tissues and is highly regulated by virtue of the inhibition of CPT-I by malonyl-CoA [3]. This intermediate is the product of acetyl-CoA carboxylase, which catalyses the first committed step in fatty acid synthesis, such that the reciprocal regulation of fatty acid synthesis and degradation is effected in liver. Malonyl-CoA is also the major physiological inhibitor of CPT-I in non-hepatic and non-lipogenic tissues, including heart [4,5].

Biochemical assays of CPT utilize four important distinctions between CPT-I and CPT-II, although catalytic activities for both are typically measured by the same forward reaction. Three characteristics are unique to CPT-I: (1) sensitivity to and binding of malonyl-CoA [3]; (2) covalent labelling and inhibition by CoA derivatives of oxirane carboxylic acids [6,7]; and (3) sensitivity to detergents [8], although this feature remains controversial [9,10]. The fourth distinguishing characteristic is molecular mass, wherein CPT-II and CPT-I have respective apparent molecular masses of approx. 70 kDa and 80–90 kDa, depending on the tissue examined. Although in dispute until recently [2,9], the malonyl-CoA sensitivity and catalytic activity of CPT-I seem to reside in a single polypeptide [10,11]. Kinetic characteristics of CPT-I differ between tissues such that muscle activity exhibits a similar Kmovan-CoA but a higher Kmavariant and a lower IC50ovan-CoA than liver activity [2].

The mammalian mitochondrial CPTs are encoded by distinct genes. The one CPT-II structural gene encodes a product of predicted molecular mass 73.8 kDa that is expressed in all fatty acid oxidative tissues [12,13]. In contrast, CPT-I is encoded by at least two genes, one of which is expressed preferentially in liver [14,15] and the other in fat and muscle [16,17]. Deduced amino acid sequences of the two isoforms exhibit 80% homology and predict proteins with almost identical molecular masses of approx. 88 kDa. However, overexpression of the rat isoforms is reported to produce proteins that migrate at different rates on SDS/PAGE, with anomalous accelerated migration of the ‘muscle’ protein proposed to account for the distinct approx. 80 kDa immunoreactive species seen in muscle and heart [18]. In this paper we refer to the CPT-I genes and gene products as α (‘liver’) and β (‘muscle’).

Multiple sequence alignment of choline/carnitine acyltransferase superfamily members [12–17,19], including the Saccharomyces cerevisiae homologues [20,21], indicates that the catalytic domains of CPT-Is reside in the C-termini of the proteins (see Figure 1A), which each include conserved His and Asp residues that are essential for catalysis [22]. The N-terminus of each CPT-I isoform includes a composite leader sequence that is retained in the mature protein [23] consisting of an intermembranous space targeting signal [24] and two 20–30-residue candidate outer-membrane-spanning/stop transfer hydrophobic domains (φ1 and φ2; see Figure 1A). The precise membrane/protein topology is the subject of debate, but malonyl-CoA binds to a locus on the cytosolic side of the outer membrane according to all models [14,23,25,26], and involves the N-terminus of the protein [25,26].

CPT-I enzyme-kinetic features in cardiac tissue are distinct from those in skeletal muscle and liver. Most notably, the IC50 of malonyl-CoA is intermediate between the nM concentration in muscle and the μM concentration in hepatic tissue. It has been proposed that this is a consequence of the co-expression of the α
and β CPT-I gene products [27]. However, more elaborate explanations are required to reconcile the brisk activity of cardiac CPT-I with the fact that cardiocyte cytosolic [malonyl-CoA] is 5 μM, or two orders of magnitude higher than a composite IC₅₀[CoA]₅ₐ₉[4,5]. In addition there is incomplete inhibitory activity of malonyl-CoA at concentrations that vastly exceed the IC₅₀ [5]. Thus we hypothesized the existence of additional CPT-I enzymes in heart. In this report we hypothesize this hypothesis and show the expression of novel isoforms of CPT-I generated by alternative splicing of the CPT-I/β gene transcript, with differing patterns of isoform enrichment in human tissues.

EXPERIMENTAL

Isolation of human CPT-I partial cDNA sequences by using PCR

Degenerate oligonucleotides corresponding to conserved heptapeptides in the mitochondrial outer membrane targeting sequence (MAEAHQQA) and within the presumed catalytic domain (WWEEYIY) of CPT-I were used in PCR with an adult human cardiac cDNA library as template (vector pCMVSPORT; BRL/Gibco). Primer sequences were: sense, 5'ggggegagaattgccacc.ATG.GCD.GAR.GCN.CAY.CAR.GC-3'; antisense, 5'ggggegagaattAT.DTA.YTC.YTC.CCA.CCA-3', exhibiting 96-fold and 24-fold degeneracy respectively. PCR products of two distinct sizes, termed hCPT-I/β and hCPT-I/β, were each cloned into pBS-KS (Stratagene) and sequenced on both strands by using the dyeose method [28].

Isolation of human CPT-I cDNA species

A radiolabelled 579 bp SalI–EcoRI restriction fragment from CPT-I/β was used as a probe in hybridization screening of the cardiac cDNA library by standard methods [28]. Positive clones obtained after secondary screening either contained a sequence identical to hCPT-I/β or contained an internal deletion and were termed hCPT-I/β. PCR primers specific for the human CPT-I ‘liver’ isoform cDNA open reading frame (ORF) [15] were used with a human liver cDNA library template (gift from Brian Seed). Primer sequences were: sense, 5'ggggegagaattgccacc.ATG.GCA.GAA.GCT.CAC.CCA.CCA-3'; antisense, 5'ggggegagaattTTA.CTT.TTT.GGA.ATT.AGA.-ACT.G-3'. The PCR product was cloned into pBS-SK to generate pBS-hCPT-I/α. Mutations revealed by DNA sequencing were corrected by replacing a 2.1 kb pBS-hCPT-I/α internal fragment of the PCR clone with a fragment from a clone subsequently isolated by hybridization screening.

Isolation of CPT-I/β genomic sequence

The genomic sequence spanning the 5' end of the CPT-I/β cDNA was amplified by ‘long-distance’ PCR with human genomic DNA and non-degenerate primers containing precise human CPT-I/β sequences. Primer sequences were: sense, 5'ggggegcgaattgccacc.ATG.GCG.GAA.GCT.CAC.CAG.GCC-3'; antisense, 5'GGT.GAT.GTA.CTC.TTC.CCA.CCA-3'. The 1.8 kb reaction product was cloned into pBS-KS and sequenced in its entirety on both strands.

Confirmation of CPT-I/β splicing forms by reverse transcriptase-mediated PCR (RT–PCR)

Total adult human cardiac, skeletal muscle or liver RNA (10 ng of each) served as templates in separate RT reactions with MuLV polymerase and an hCPT-I/β-specific anti-sense primer RTβ1 (5'TTG.CTG.TTC.ACC.ATG.AGA.GGG.CT-3') that hybridizes 3' to all of the alternative splice junctions in the hCPT-I/β forms detected. RT products were further analysed with isoform-specific anti-sense primers in combination with the common sense strand primer Fβ1(5'ATG.GCG.GAA.-GCT.CAC.CAG.GCC-3') in separate PCRs. Isoform-specific primers were: RTβ1 + β3, 5'GGGGGATCTCCTAGTAC-GAA-3'; RTβ2, 5'AGAAAGGCGGATACACATCAG-3'; RTβ3, 5'CGCCAGACCTTAGGTAAGGC-3', where 3' residues that are specific for the isoform splice junction are indicated in bold. Products of each reaction were separated by agarose-gel electrophoresis after various PCR cycle increments, and revealed with ethidium bromide staining.

Ribonuclease protection assays (RPAs)

SalI–NcoI and NcoI–EcoRI fragments of hCPT-I/β1, a SalI–HindIII fragment from hCPT-I/β2, SalI–EcoRI fragments from hCPT-I/β1 and β3, and an AgeI–NcoI fragment from the 3' end of the hCPT-I/β ORF were each cloned into pBS such that the 3' end of the cDNA fragment was apposed to the vector T7 promoter. cRNA probes were prepared by standard procedures in accordance with the instructions of the RPA kit manufacturer (Ambion). In brief, after plasmid template linearization by digestion with XhoI, cRNA probes were generated with T7 RNA polymerase and ATP, CTP, GTP and [α-32P]UTP (800 Ci/mmol; New England Nuclear). Denaturing PAGE-purified probes [2–10]×10⁶ c.p.m.] were hybridized at 42°C for 16 h with 2–10 μg of total RNA isolated from adult human heart, skeletal muscle or liver (Clontech); samples were digested with RNase A and RNase T₁, and reaction products were resolved by using 6% denaturing PAGE. Parallel lanes were loaded with 32P-end-labelled DNA standards. Results were detected by autoradiography, and relative amounts of the mRNA species were determined by densitometric analysis of bands corresponding to each protected fragment and by scintillation counting of bands excised from gels, corrected for the specific radioactivity of each probe as determined by the total number of U residues in each sequence.

RESULTS

Identification of novel human CPT-I cDNA species

A PCR strategy was employed to evaluate our hypothesis that the observed unique enzyme kinetic behaviour of CPT-I in heart might be accounted for by the co-expression of unique isoform(s). Our focus was directed by the behaviour of this enzyme with respect to inhibition by malonyl-CoA, an activity that resides at the N-terminus of the polypeptide on the basis of the location of two hydrophobic candidate membrane-spanning domains (Figure 1A, φ1 and φ2) [14,23,26]. Thus PCRs with a human cardiac cDNA library template used pairs of degenerate primers hybridizing with coding sequences that flanked this region and were conserved across species and CPT-I isoforms. Degenerate oligonucleotide primers corresponding to conserved heptapeptides in the mitochondrial targeting sequence (MAEAHQQA) and within the presumed catalytic domain (WWEEYIY) of CPT-I produced PCR products of two distinct sizes. Each purified PCR product gave only a single band on reamplification with the original primers, excluding internal priming by the degenerate primers as an explanation for the multiple bands. On sequence analysis, the longer product showed complete identity with the previously identified human CPT-I ‘muscle’ form [17],
Carnitine palmitoyltransferase I mRNA splicing variants

Figure 1 Structure of carnitine acyltransferases

(A) Diagram of the amino acid sequence alignment of rat carnitine octanoyltransferase (rCOT), and human CPT-II, CPT-Iα1 and CPT-Iβ1. The deduced COT sequence is modified from the original report [19] as suggested subsequently [20]. The respective N-terminal peroxisomal and mitochondrial matrix targeting sequences of COT and CPT-II are indicated by hatched boxes. N-terminal hydrophobic domains of the CPT-I isoforms are indicated by φ1 and φ2. Percentage amino acid similarities to hCPT-Iβ within demarcated regions of each other enzyme sequence are shown.

(B) Degenerate primers based on the conserved CPT-I amino acid sequences depicted were used in PCR with a human cardiac cDNA library template. Products of two different lengths were isolated, representing partial cDNA species of hCPT-Iβ1 and hCPT-Iβ2. hCPT-Iβ3, a third cDNA, was detected by hybridization screening of the library. Deletions within the β2 and β3 sequences compared with β1 are depicted. Hydrophobicity plots based on Kyte and Doolittle [40] for the deduced protein sequence of each CPT-Iβ isoform are shown, with the hydrophobic φ1 and φ2 regions noted. φ1* indicates the unique hydrophobic domain of hCPT-β2.

whereas the shorter sequence contained a 210 bp internal deletion but was otherwise identical. The sequence at the 5′ end of the deleted segment was gtcagt (encoding Val-Ser in the long form), which diverges by only a single residue from the consensus splice donor sequence [29], suggesting that this shorter sequence had an authentic cognate mRNA generated by use of this cryptic donor signal. These cDNA sequences were termed hCPT-Iβ1 and hCPT-Iβ2 respectively.

The hCPT-Iβ1 fragment obtained by this technique was then used in hybridization screening of the human cardiac cDNA library. Three out of six positive clones obtained after secondary screening were essentially identical with the previously reported full-length clone [17] containing the CPT-Iβ1 coding sequence. Three additional positive clones contained a distinct internal 102 bp deletion. As depicted schematically in Figure 1(B), the 5′ end of the sequence deleted from these clones was immediately adjacent to the 3′ end of the sequence deleted from hCPT-Iβ2, suggesting that this was a splice junction and further supporting the authenticity of both as cDNA species. This sequence was termed hCPT-Iβ3.

Partial characterization of the human CPT-Iβ gene structure

The genomic sequence spanning the 5′ end of the CPT-Iβ cDNA was amplified by using human genomic DNA and primers containing hCPT-Iβ sequences in long-distance PCR. This 1.8 kb reaction product sequence was identical with a recently isolated human chromosome 22q13 sequence [30]. Figure 2(A) is a schematic depiction of the hCPT-Iβ gene structure in the region relevant to this work. The exon containing the initiation codon is numbered 2 because there are two additional alternatively used 5′ exons [31]. This analysis verified that the cryptic splice donor in exon 3 is used to generate β2 mRNA and that sequences omitted from the β2 and β3 mRNA species flank a splice junction between exons 4 and 5. Splicing sites used by each of the alternative isoforms are noted in Figure 2(B); all conform to the -gt and yag- consensus donor and acceptor splice sites respectively [29].

Verification of novel CPT-Iβ mRNA species by RT–PCR

We used RT–PCR to evaluate the authenticity of these mRNA splicing forms. Adult cardiac, skeletal muscle and liver total RNA served as templates in RT with an hCPT-Iβ-specific antisense primer that hybridized 3′ to all of the alternative splice junctions in the hCPT-Iβ forms detected (Figure 3A; RTβ_common). RT products were further analysed by using isoform-specific anti-sense primers, in combination with a common sense-strand primer that hybridized near the initiation codon (Figure 3A;
Figure 2  Partial genomic structure and splicing sites of human CPT-Iβ

(A) Schematic representation of the hCPT-Iβ gene in the region of alternative splicing. The exon containing the initiation codon is numbered 2 because there are two alternatively used untranslated 5′ exons [31]. (B) Splice donor and acceptor sequences used to generate the three hCPT-Iβ isoforms. Nucleic acids within exons are shown in capital letters, intron sequences are shown in lower-case letters, and splice donor and acceptor sequences are underlined. Residues within these sites that conform to the (gtragt) and (ynynag) consensus for donor and acceptor [29] are in bold type.

Fβ_common). Isoform-specific primers were designed to hybridize either with a sequence entirely unique to forms (RTβ1–3), or to contain residues at the 3′ end that span the isoform-specific splice junction (RTβ1, RTβ3). Control reactions with each isoform cDNA as template were performed to verify primer specificity. By using this technique the presence of all three hCPT-Iβ mRNA species was confirmed in human heart and skeletal muscle as well as in liver. Each RT primer gave a PCR band of appropriate size to confirm the use of the specific alternative splice junction (Figure 3B). No additional bands were detected, indicating that no other significant splicing variants were present in these samples that deleted exons between Fβ_common and the RTβ primers. Differential tissue enrichment of the splicing variants was suggested by semi-quantitative analyses of the PCR data (see below).

Verification and quantification of novel CPT-Iβ mRNA species by RPA

We utilized RPA to verify the existence of these CPT-Iβ isoform mRNA species, to seek other splicing variants and to quantify relative levels of isoform expression in human heart, skeletal muscle and liver. Because CPT-Iz is known to be expressed in liver and co-expressed in heart, we also included an hCPT-Iz-specific probe in some of these experiments. We have also detected alternative splicing forms of this gene at the 5′ end of the coding region (G.-S. Yu, Y.-C. Lu and T. Gulick, unpublished work), so that this probe corresponded to the 3′ end of the ORF, enabling detection of a single protected CPT-Iz mRNA fragment. Figure 3(A) illustrates the cRNA probes used in the RPA studies, as well as the predicted lengths of the protected mRNA fragments.

By using RPA, all of the CPT-Iβ splice variant mRNAs were detected in heart, skeletal muscle and liver RNA. Each cRNA probe protected a fragment of appropriate length to verify the presence of the mRNA species from which the probe was derived, seen as the slowest migrating band in each lane in Figure 3(C), panels 1 to 3. As expected, each probe also protected fragments corresponding to the other isoform mRNA species, although in some cases the shorter fragments were detected only with longer autoradiography exposure times (bands identified in square brackets in Figure 3C). The multiple bands present at 65–67 bp in Figure 3(C), panel 2, do not reflect splicing slippage but are instead due to variable RNase digestion of the two U/A bp at the end of this cRNA probe derived from the HindIII restriction site (AAGCTT) within the cDNA sequence. Densitometric analyses of the isoform-specific bands in multiple experiments showed that the β1 mRNA was 80–90% of total CPT-Iβ mRNA in both heart and muscle samples, whereas β2 and β3 each contributed 5–10% to the total.

Additional RPAs were performed to characterize the splicing variants further. Although the RPA/β1 and RPA/β3 probes each definitively verified the existence of the three splicing variants, both seemed to protect additional band(s), in contrast with the RPA/β2 probe. The β1 and β3 probes each contained two sequence stretches that were not present in the β2 probe, thereby defining candidate regions for additional mRNA splicing sites. We performed RPAs with two additional probes spanning these regions (RPA/β1B and RPA/β1C; Figure 3A). The β1B probe, derived from the 5′ end of this sequence, protected two fragments in each tissue RNA sample of appropriate size to account only
Figure 3 Alternatively spliced forms of CPT-Iβ are expressed in human heart, muscle and liver

(A) Locations of probes and primers used in RT–PCR and RPA analyses are superimposed on diagrams of partial CPT-I mRNA sequences. Splice junctions are indicated by white lines. Lengths of PCR products or protected mRNA fragments are indicated. A potential additional cryptic splice donor in exon 6 is indicated by white asterisks. 

(B) RT was performed with primer R1common and human cardiac, skeletal muscle and liver total RNA. These reactions served as templates in PCR with primer Fβcommon with each of the isoform-specific RT primers in separate reactions. PCRs were retrieved after the indicated cycles and products were separated by agarose-gel electrophoresis. Products of control reactions with the β isoform cDNA species as templates were separated in the three lanes adjacent to the DNA standards (bp) on the left. 

(C) RPAs were performed with human cardiac (H), skeletal muscle (M) and liver (L) total RNA with cRNA probes shown in (A). Autoradiograms of electrophoresed reaction products for each probe are shown, and protected fragments corresponding to each isoform are indicated at the right of each panel. Bands that required longer autoradiography exposure times are indicated in square brackets, and those indicated by asterisks could each be explained by the use of a cryptic splice donor site in exon 6. Standards and their sizes (in nt) are shown on the left of panels 1, 4 and 5.

for the isoforms already identified (results not shown). Fragments protected by the β1c probe, which spanned the exon 4/5 and 5/6 junctions, were consistent with the existence of the β1, β2 and β3 mRNA species and the use of at least one additional significant alternative splice site (Figure 3C, panel 4). Although we have yet to characterize this message, it seems to result from use of a cryptic splice donor sequence near the 3' end of exon 6. This location is indicated in Figure 3(A) by asterisks, and the corresponding protected fragments are similarly marked in Figure 3(C). Protected fragments are consistent with use of this
splice donor in combination with alternative splicing that generates each of the \( \beta_1, \beta_2 \) and \( \beta_3 \) messages, implying that there are at least three additional mRNA products of the CPT-I/\( \beta \) gene that encode different ORFs.

We used RPA to evaluate the relative levels of CPT-Iz and CPT-I/\( \beta \) mRNA expression in human heart, muscle and liver. In these studies we used the \( \beta_2 \) and \( \alpha \)-specific probes in co-hybridization with each tissue RNA. The RPA probe, derived from the 3' end of the cDNA ORF, protected a single mRNA fragment in the tissue RNA samples, confirming a lack of splicing variation in this region (Figure 3C, panel 5). These assays unexpectedly revealed expression of the \( \alpha \) isoform mRNA in each of these samples, including skeletal muscle. The co-hybridization experiments demonstrated that the \( \alpha \) message was predominant in liver, whereas the \( \beta \) mRNA was, as expected, predominant in the heart and muscle samples. Relative isoform mRNA abundances in these tissue samples could be further characterized as follows: liver, \( [\alpha] \geq [\beta_1] > [\beta_2] > [\beta_3] \); skeletal muscle, \( [\beta_1] > [\beta_2] = [\beta_3] > [\alpha] \); heart, \( [\beta_1] > [\beta_2] = [\beta_3] = [\alpha] \). Of particular note, the novel splicing variants described here were present in equal abundance to that of the CPT-Iz mRNA in heart, and all of the isoforms were expressed in each of these tissues.

**DISCUSSION**

We have demonstrated the existence of two novel splicing forms of one human CPT-I gene transcript, referred to as hCPT-Iz/2 and \( \beta_3 \), generated by the use of a cryptic splice donor site within an exon and/or by exon skipping. RT-PCR and RPA, which are more sensitive than the Northern blotting experiments performed by others, reveal that these splicing forms and the previously identified CPT-Iz/1 are expressed in skeletal muscle and liver as well as in heart, and further that CPT-Iz is also expressed in all of these tissues, albeit at different absolute and relative levels. Although the novel isoforms represent a relatively small fraction of the total CPT-I mRNA in these samples, their potential importance relates to the deduced protein sequences, which have internal deletions that predict novel features compared with the two previously identified CPT-I isoforms [14–18].

These CPT-I sequences were detected in four sources of normal human tissue, including three tissue RNA samples and an independent cDNA library. Together with the existence of three analogous CPT-I/\( \beta \) mRNA species in rat tissues (G.-S. Yu and T. Gullick, unpublished work), this is strong evidence that these messages are authentic normal products of this gene. The lack of previous detection of these mRNA species is consistent with the small difference in respective sizes compared with the approx. 3.0 kb CPT-Iz/1 message, such that they would not be distinguished in standard RNA blotting assays.

Although we have yet to demonstrate definitively that the alternatively spliced mRNA species produce enzyme isoforms in these tissues, there are several lines of evidence to support their existence. First, the three CPT-I/\( \beta \) isoforms described contain identical 5' untranslated regions and ribosome-binding sites. Because this is the major determinant of translation efficiency [32], we expect that the CPT-I/\( \beta \) isoform proteins will be present in proportion to the respective mRNA abundance. Secondly, our analyses were performed on total RNA samples; thus any differences in mRNA stability between isoforms were accounted for. Again, there are reasonable explanations for a lack of detection in earlier analyses of tissue enzyme. CPT-I purification and identification techniques have assumed a single entity with stereotypical labelling and binding activities and membrane association. It is likely that the novel forms exhibit unique characteristics in some of these respects, such that they might not co-purify, label or otherwise behave in a manner detectable by these techniques. Further, other investigators might have attributed minor activities to degradation products, CPT-II or non-specific sources. Finally, we and others [18,33] have found that the CPT-I/\( \beta \)1 protein exhibits anomalous accelerated migration on SDS/PAGE, which might interfere with the ability to segregate isoforms with small differences in molecular mass. In this regard, CPT-I/\( \beta \) isoforms translated in vitro migrate more closely to one another on SDS/PAGE than would be predicted from the respective deduced sizes (G.-S. Yu and T. Gullick, unpublished work). Definitive proof of the presence of CPT-I/\( \beta \), \( \beta_3 \) and \( \beta_1 \) enzyme isoforms in human tissues will require specific antibodies and/or two-dimensional electrophoretic separations.

It has long been established that CPT-I activity is associated with the outer mitochondrial membrane [1,2,25]. A case has been made that the malonyl-CoA inhibitory and CPT catalytic activities reside on opposite sides of this barrier [25]. However, the primary structures of CPT-Iz/2 and CPT-Iz/1 each include two hydrophobic stretches, and recent experimental results for rat liver CPT-I indicate that both are used to span the outer membrane [26]. This dictates that both the N- and C-termini and CPT-Iz are located on the cytosolic side of the outer membrane [26]. Because of analogous hydrophathy profiles and predicted secondary structure in this region, CPT-Iz/1 is expected to exhibit similar topology. As all three CPT-I/\( \beta \) mRNA encode identical leader sequences, it is virtually certain that their respective products are all directed to the mitochondrion. However, the deduced CPT-Iz/2 isoform sequence has only a single hydrophobic domain, such that this isoform is predicted to exhibit novel membrane/protein topology compared with the other forms. We propose that multiple products of this gene share a common active site but that respective precise subcellular loci and membrane association properties differ, perhaps reconciling the conflicting results of other investigators [25,26].

Differing membrane/enzyme topologies of CPT-I isoforms have an impact on the binding of substrate(s) and/or competitive inhibitors such as the oxirane carboxylates [2,6,7,34]. Owing to partitioning between the cytosol and mitochondrial intermembranous space, catalytic efficiency in terms of the delivery of acylcarnitine to the inner membrane translocase might differ between these isozymes, despite identical catalytic domain structures. In any case our demonstration that all four isoforms are expressed in three different tissues bears on efforts to identify tissue- and isoform-specific CPT-I inhibitors, because previous efforts have focused exclusively on CPT-Iz/1 and \( \beta_1 \) [2,34].

The high-affinity malonyl-CoA allosteric regulatory site of CPT-I [35] has not been mapped precisely. However, the N-terminus seems to dictate the sensitivity of CPT-I to this inhibitor [11] through interactions with the C-terminal active site [26], and inhibition clearly has an allosteric component [2,4,5,35]. Such intramolecular interactions, as well as those between CPT-I enzyme and the membrane that might affect catalytic or inhibitory activities [36], are likely to be different for CPT-Iz/3 compared with CPT-Iz/1, and are necessarily different for CPT-Iz/2. We therefore speculate that the novel \( \beta \) isoforms will also exhibit unique kinetic behaviours with respect to allosteric regulation.

Our results confirm the preferential expression of CPT-Iz in liver and of CPT-I/\( \beta \) in skeletal muscle, with isoform co-expression in heart. However, differential relative abundances of the CPT-I/\( \beta \) isoforms across tissues are also apparent. We were surprised to note similar relative levels of \( \beta \) isoform expression in skeletal muscle and heart, but we attribute this to co-isolation of fast-
and slow-twitch skeletal muscle RNA, with the contribution of the latter rendering the samples similar to the cardiac RNA. It is also possible that different cell types within the tissues examined contribute specific mRNA species or have differing relative contributions to total tissue CPT-Iβ mRNA. The mechanism of this differential enrichment is obscure, but might provide for the regulated expression of the splicing variants, which we hypothesize will contribute to changes in tissue CPT-I enzyme activity seen during development [37] and in response to hormonal stimuli and metabolic status [2,33,38,39], along with other activity seen during development [37] and in response to hormonal stimuli and metabolic status [2,33,38,39], along with other factors [36].

In summary, multiple alternative splicing forms of the human CPT-Iβ gene transcript are differentially expressed in tissues with high rates of fatty acid β-oxidation. Two novel mRNA species encode enzymes that have internal deletions within their respective N-termini compared with the previously identified CPT-I enzymes. We propose that the composite regulated expression of these and other products of alternative mRNA splicing are responsible for CPT-I enzyme activities in tissues, and that this might provide a molecular mechanism for observed myocardial CPT-I activities [4,5] as well as the variations in tissue CPT-I kinetics seen in other tissues [2,38].

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