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The Mitochondrial Tricarboxylate Carrier

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The tricarboxylate carrier has recently been purified from rat liver mitochondria by three distinct scientific groups using different methods. A 37–38-kDa protein has been prepared by silca gel 60 chromatography by our group (Claeys and Azzi, 1989; Glerum *et al.*, 1990). The specific citrate transport activity of this preparation is not significantly different from that measured in mitochondria and it is inhibitable by 1,2,3- benzenetricarboxylic acid. Bisaccia *et al.* (1990) have reported the isolation of a 30-kDa protein by Celite 535 chromatography, and Kaplan's group (Kaplan *et al.*, 1990) have isolated a 32.5-kDa protein by Matrex Orange, Matrex Blue, and Affi-Gel chromatography. Peptide mapping has failed to support any structural homologies between the 37–38-kDa and the 30–32.5-kD proteins. The 38-kD protein is N-terminally blocked. The peptides obtained by several cleavage procedures have been partially sequenced. Their sequence information has been used to obtain different cDNA clones by a dual approach, the polymerase chain reaction and screening of a λZAP cDNA library. The largest cDNA which could be isolated is 2,986 bp in length and contains a 1071-bp-long open reading frame and an unusually long 3′ untranslated region, both of which have been completely sequenced. The protein sequence of the carrier from the first in-frame methionine is 322 amino acids in length and exhibits a molecular mass of 35,546. Comparison of the protein sequence to the sequences of the four members of the mitochondrial carrier protein family (ADP/ATP carrier, phosphate carrier, 2-oxoglutarate/malate carrier, and uncoupling protein) does not reveal significant similarity (cf. Walker *et al.*, 1987). A tripartite internal homology, which is a characteristic of these proteins, is not present in the sequence of the tricarboxylate carrier protein. The mRNA for the tricarboxylate carrier is expressed in rat liver and brain, but not in rat heart.

KEY WORDS: Tricarboxylate carrier; mitochondrial transport; citrate.

INTRODUCTION

The tricarboxylate carrier, one of the mitochondrial anion transporters (LaNoue and Schoolwerth, 1979), was discovered by Chappell and Haarhoff (1967) in liver mitochondria. Citrate, *cis*-aconitate (but not *trans*-aconitate), threo-D-isocitrate, D- and L-tartrate, malate, phosphoenolpyruvate, and succinate are substrates for this carrier. α-ketoglutarate and malonate are not transported by the tricarboxylate carrier (Robinson *et al.*, 1971). 1,2,3-Benzenetricarboxylic acid (BTA) is the best characterized inhibitor of the carrier. Butylmalonate and *N*-ethylmaleimide are much less potent inhibitors of the transport of dicarboxylic acids by the tricarboxylate carrier, than by the dicarboxylate carrier, suggesting that the di- and tricarboxylate carriers are different molecular entities (Palmieri *et al.*, 1972). The synthesis of fatty acids and cholesterol, which takes place in the cytosol, begins with acetyl-CoA. Acetyl-CoA derives from the mitochondrial citrate transported to the cytosol, via the tricarboxylate carrier (see Fig. 1). Long-chain acyl-CoA esters, as inhibitors of citrate transport, may exert a significant control of fatty acid and cholesterol biosynthesis (Halperin *et al.*, 1972). Coleman and co-workers (cf. Coleman, 1986) have observed an activating effect of

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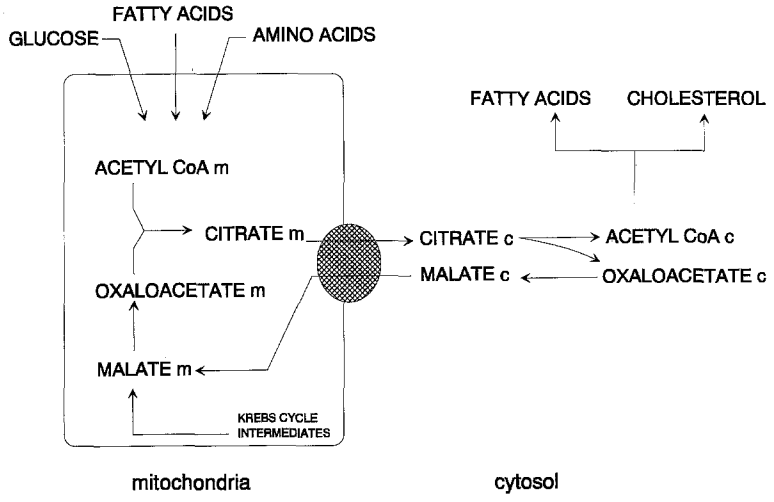


Fig. 1. The role of citrate in cell metabolism (modified from Coleman, 1986).

cholesterol on the tricarboxylate carrier of tumor mitochondria.

The tricarboxylate carrier also plays a role in the gluconeogenesis and in the shuttle of reducing equivalents across the mitochondrial membrane, since both reactions require an effective mitochondrial transport of citrate and phosphoenolpyruvate (for a review, see Robinson, 1972).

The tricarboxylate carrier has been most widely studied in liver mitochondria, although it is also found in brain and kidney mitochondria, albeit with an apparently lower activity. No or little carrier activity is present in heart mitochondria (Sluse *et al.*, 1971).

The metabolite carriers sequenced thus far have been proposed to form a family (cf. Palmieri *et al.*, 1992). Original sequence studies of the adenine nucleotide (Aquila *et al.*, 1982) and phosphate (Aquila *et al.*, 1987) carriers, along with the uncoupling protein (Aquila *et al.*, 1985), have demonstrated the presence of a tripartite structure with internal homology. Each of these proteins consists of three related segments about 100 amino acids in length, which are proposed to form two transmembrane helices joined by a hydrophilic extramembrane loop (Runswick *et al.*, 1987). The recent cloning of the oxoglutarate/malate carrier has added a new member to the family of the carrier proteins (Runswick *et al.*, 1990). This has thus raised the question whether the other metabolite carriers will be related to this family, or whether there will be several families within the group of mitochondrial transporters. A further goal of this study has thus been to compare the cloned cDNA encoding the rat liver mitochondrial tricarboxylate carrier previously isolated in our laboratory (Glerum *et al.*, 1990) with the known carrier sequences. Here we show that the 322 amino acid sequence does not show any direct similarity with the members of the carrier family analyzed thus far, nor does it show the tripartite internal homology characteristic of the carriers previously analyzed.

PURIFICATION OF THE TRICARBOXYLATE CARRIER

Purification of a bovine or rat liver mitochondria Triton X-100 extract by hydroxyapatite and silica gel chromatography results in a reconstitutively active tricarboxylate carrier (Claeys and Azzi 1989; Glerum *et al.*, 1990). This purification yields a single protein band as detected in SDS-PAGE (Fig. 2). The two preparations from rat and bovine liver differ by about 1 kDa in molecular mass. The higher apparent molecular mass of the protein from rat liver could represent a species-specific form of the tricarboxylate carrier.

Figure 3 shows the results of experiments indicating that the substrate transport specificity of the isolated bovine liver carrier is similar to that measured in intact mitochondria. Figure 4 shows that the half-inhibiting concentration of the carrier by 1,2,3-benzenetricarboxylic acid is similar to that obtained with intact liver mitochondria.

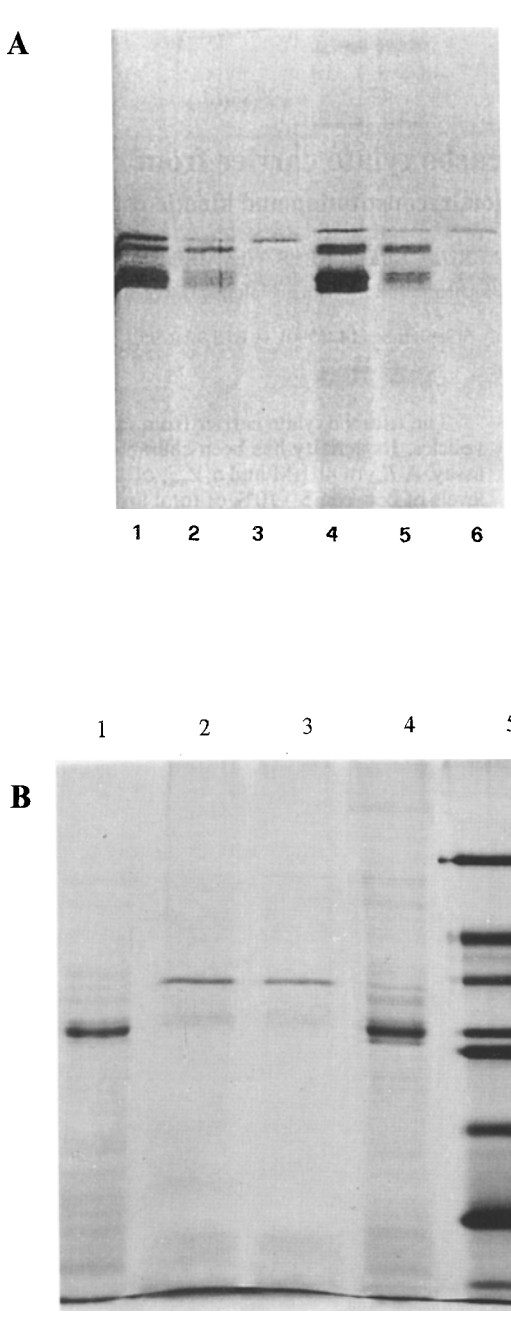


Fig. 2. (A) Polypeptide pattern of the different purification steps for the tricarboxylate carrier. SDS-PAGE and silver staining were carried out as described (Glerum *et al.*, (1990). Lanes 1 and 4, hydroxyapatite eluate; lanes 2 and 5, fraction not bound to silica gel; lanes 3 and 6, silica gel eluate. Lanes 1–3 represent the purification from bovine liver mitochondria, and lanes 4–6 from rat liver mitochondria (from Azzi *et al.*, 1993). (B) Preparation of the tricarboxylate carrier from mitoplasts. Lane 1 and 2 correspond to mitoplasts HTP and silica gel preparations, lane 3 and 4 correspond to mitochondria HTP and silica gel preparations, and lane 5 corresponds to Mw standards (from top 66, 45, 38, 29, 24, 20.1, 14.2 kDa, respectively) (from Azzi *et al.*, 1993).

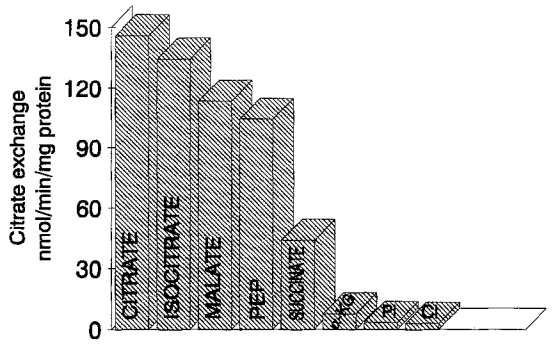


Fig. 3. The substrate transport specificity of the isolated tricarboxylate carrier. Activity measurements were performed as described by Lüthy and Azzi (1989).

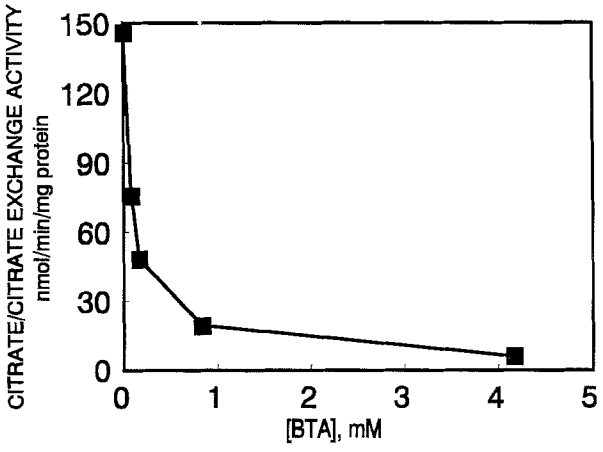


Fig. 4. Benzenetricarboxylic acid inhibition of citrate transport by the isolated tricarboxylate carrier. Activity measurements were performed as described by Lüthy and Azzi (1989).

ISOLATION OF THE TRICARBOXYLATE CARRIER FROM THE INNER MITOCHONDRIAL MEMBRANE

To ensure that the 38-kDa protein was indeed a protein of the inner mitochondrial membrane and not a constituent of either peroxisomal or lysosomal membranes, which are known to copurify with mitochondria, we have purified the 38-kDa protein from mitoplasts prepared by the digitonin method. This result (Fig. 2B) shows that the protein is localized in the inner mitochondrial membrane and cannot be ascribed to the outer membrane or to other organelles known to contaminate mitochondrial preparations.

Table I. Differences between Three Different Preparations of the Tricarboxylate Carrier

Procedure after HTP	Silica gel	2 Dye columns	Celite
Detergent	Triton X-114	Triton X-114	Triton X-100
Mr	38,000[a]	32,500	30,000
Activity (nmol/min/mg)	1,625	810	1,350
Inhibition by:			
BTA (1 mM)	79%	74%[c]	96%
Phenylsuccinate (2 mM)	29%	−5%	48%
Phenylglyoxal (2 mM)	59%	100%	
NEM[b] (0.2 mM)	26%	79%	16%
Cyano OH cinnamate (1 mM)	72%	-2%	

[a] *Mr* of the rat protein. A *Mr* of 37,000 was found for the bovine protein.
[b] Low inhibition in mitochondria.
[c] 2 mM.

COMPARISON OF DIFFERENT TRICARBOXYLATE CARRIER PREPARATIONS

Bisaccia *et al.* (1990) have reported the isolation of a 30-kDa protein by Celite 535 chromatography, and Kaplan *et al.* (1990) have isolated a 32.5-kDa protein by Matrex Orange, Matrex Blue, and Affi-Gel chromatography. Both preparations are provided with citrate transport activity. Table 1 shows that the three preparations have similar specific activities and that they are equally sensitive to 1,2,3-benzenetricarboxylic acid. The difference in molecular mass appears, however, significant and cannot be caused by dissimilar amounts of detergent or phospholipid remaining associated with the protein after purification. In order to ascertain whether the lower-molecular-weight polypeptides from Kaplan's (Kaplan *et al.*, 1990 & 1991) and Palmieri's (Bisaccia *et al.*, 1990) laboratories originated from proteolytic digestion of a higher-molecular-weight precursor, such as the protein obtained in our laboratory, the sensitivity of the preparations to endoproteinase Glu-C and to chemical splitting were analyzed.

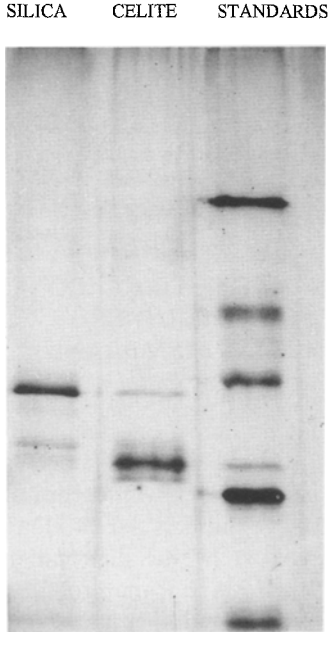


Fig. 5. Comparison between celite (Bisaccia *et al.*, 1990) and the silica gel preparations (Glerum *et al.*, 1990), as obtained in our laboratory, upon analysis by SDS-PAGE (from Azzi *et al.*, 1993).

Figure 5 shows the comparison between the Palmieri and the silca gel preparations, as prepared in our laboratory, upon analysis by SDS-PAGE. The results suggest that the difference in molecular mass between the two preparations are not due to differences in detection methods, but are rather intrinsic properties of the polypeptides isolated.

Figure 6 shows that the silica gel preparation was split into two fragments by the endoproteinase Glu-C, the peptides obtained being 18 and 20 kDa in molecular mass, respectively. The latter could be partially sequenced (see below), while the former was blocked at the amino terminus. The preparation of Palmieri's group (Bisaccia *et al.*, 1990), in contrast to ours (Glerum *et al.*, 1990), was not split by endoproteinase Glu-C. Cleavage of the silica gel protein at asparagine–glycine bonds with hydroxylamine produces two fragments, of 14 and 24 kDa masses, respectively. The former fragment could again be partially sequenced (see below), while the latter was blocked at the amino terminus. Also in this case, no splitting was obtained with the Celite preparation (Fig. 7). Finally, CNBr fragmentation produced a

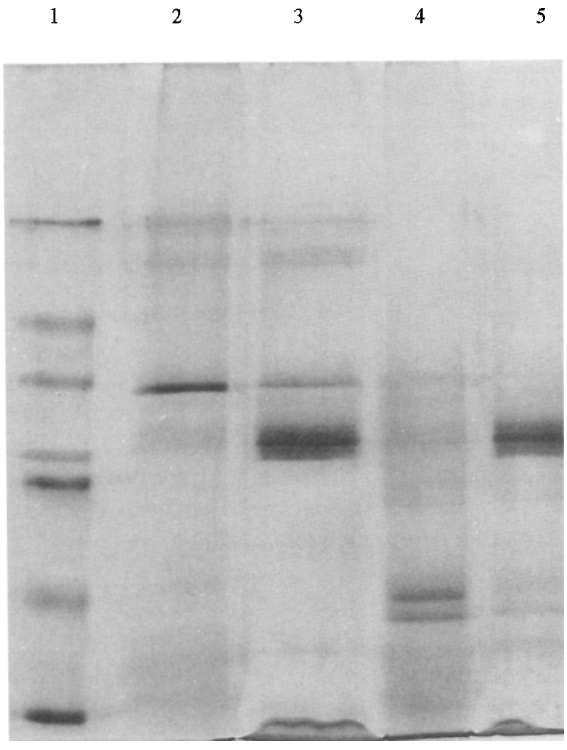


Fig. 6. Splitting of the silica gel preparation into two fragments by the endoproteinase Glu-C. Lane 1, Mw standards; Lanes 2 and 4, silica gel preparation before and after endoproteinase Glu-C splitting, respectively; Lanes 3 and 5, Celite preparation before and after endoproteinase Glu-C splitting, respectively (from Azzi *et al.*, 1993).

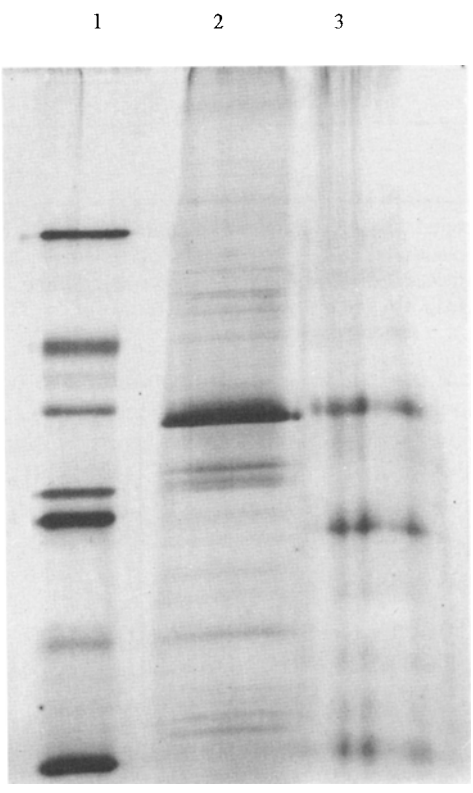


Fig. 7. Cleavage of the silica gel protein (but not of the Celite preparation) with hydroxylamine produces two fragments. Lane 1, Mw standards; Lane 2, silica gel preparation; Lane 3, hydroxylamine fragments (from Azzi *et al.*, 1993).

large number of peptides of small molecular weight from the silica preparation, and a major fragment of molecular mass 24 kDa with the Celite preparation.

In conclusion, the two preparations from Celite and silica gel are structurally different, and the former cannot be considered a proteolytic degradation product of the latter. On the basis of the similarities pointed out by Kaplan (Kaplan *et al.*, 1990), between their preparation and that of Palmieri's group (Bisaccia *et al.*, 1990), it can be inferred that Kaplan's preparation is likewise not a degradation product of the preparation obtained in our laboratory.

PROTEIN SEQUENCE ANALYSIS OF THE TRICARBOXYLATE CARRIER

Protein sequence analysis of the intact carrier revealed that the N-terminus is blocked, though the nature of the modification is unknown. Through enzymatic digestion and chemical cleavage, partial

Table II. N-Terminal Amino Acid Sequences of Some Peptides Obtained from the Tricarboxylate Carrier

CNBr fragment	...QREL?VGIP VTDEN**GTRLG E**.....
Hydroxylamine fragment	..**GTRLG** ESTNAAKQAITQVVISRIL
Endoproteinase Glu-C	..LGTAYVSA TTGAVATALG ?NA?T
CNBr fragment	?VTSLE DDLQASIQ?S ?PELR?VYF

Table III. Two of the Oligonucleotide Sequences Used as Primers in the PCR Reactions

Name[b]	Peptide sequence	Primer sequence[c]	Redundancy
cc10	VTDENGT	AGCTGAATTCGTXACDGATGARAATGCXAC	96
cc11 (r)	KQAITQV	ACGTAAGCTTACYTGXGTRATXGCYTGYTT	256

[a]The peptide sequences are those used for the design of the oligonucleotides. Forward primers were constructed with an EcoRI-site at the 5′-end, and reverse primers with HindIII-site (Glerum *et al.*, 1990).
[b](r) indicates that the primer is in the reverse direction.
[c]R stands for A or G, Y for C or T, D for A or G or T, and X for A or C or G or T.

internal protein sequence information was obtained, as shown in Table II. The sequence of one hydroxylamine-generated peptide overlapped with one obtained by CNBr cleavage (cf. Table II). These sequences were all found in the full-length cDNA clone obtained, as shown in Fig. 9.

ISOLATION OF RAT LIVER cDNA CLONES FOR THE MITOCHONDRIAL TRICARBOXYLATE CARRIER

A series of oligonucleotide primer mixtures was synthesized for use in polymerase chain reactions. The combination of cc10 as forward primer and cc11 as reverse primer (Table III) yielded, based on the peptide sequence of one of the CNBr fragments and the overlapping hydroxylamine generated fragment (Table II), the expected 88-bp product comprising a 30-bp stretch of unique sequence flanked by the two regions originating from cc10 and cc11. The unique 30-bp sequence was used to synthesise a 30-mer oligonucleotide (ccr1), which was used to screen approximately 1 × 106 plaques of a rat liver cDNA library. A clone of 2.0 kb (clone 16, 124 amino acids) contained both sequences obtained from the CNBr cleavage as well as that determined from the hydroxylamine splitting. Using an EcoRI-HindII fragment (300 bp) of this cDNA as probe in a further round of screening, one of the positive clones (164) extended 384-bp 5′ of clone 16 and had a poly (A) tail (Fig. 8). Using a 258-bp EcoRI-NArI fragment as probe from the coding region of clone 164, a full-length cDNA clone was isolated (116-16).

NUCLEOTIDE SEQUENCE OF TRICARBOXYLATE CARRIER cDNA

The complete cDNA sequence is shown in Fig. 9, and was determined in both directions. The nucleotide sequence is 2,986 bp long, with 1071 bp constituting a

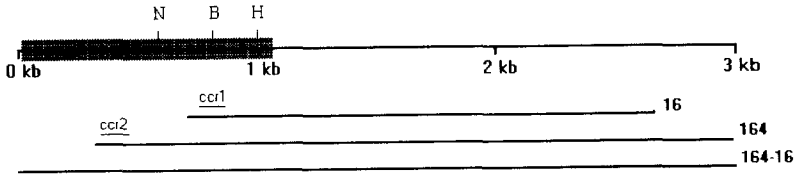
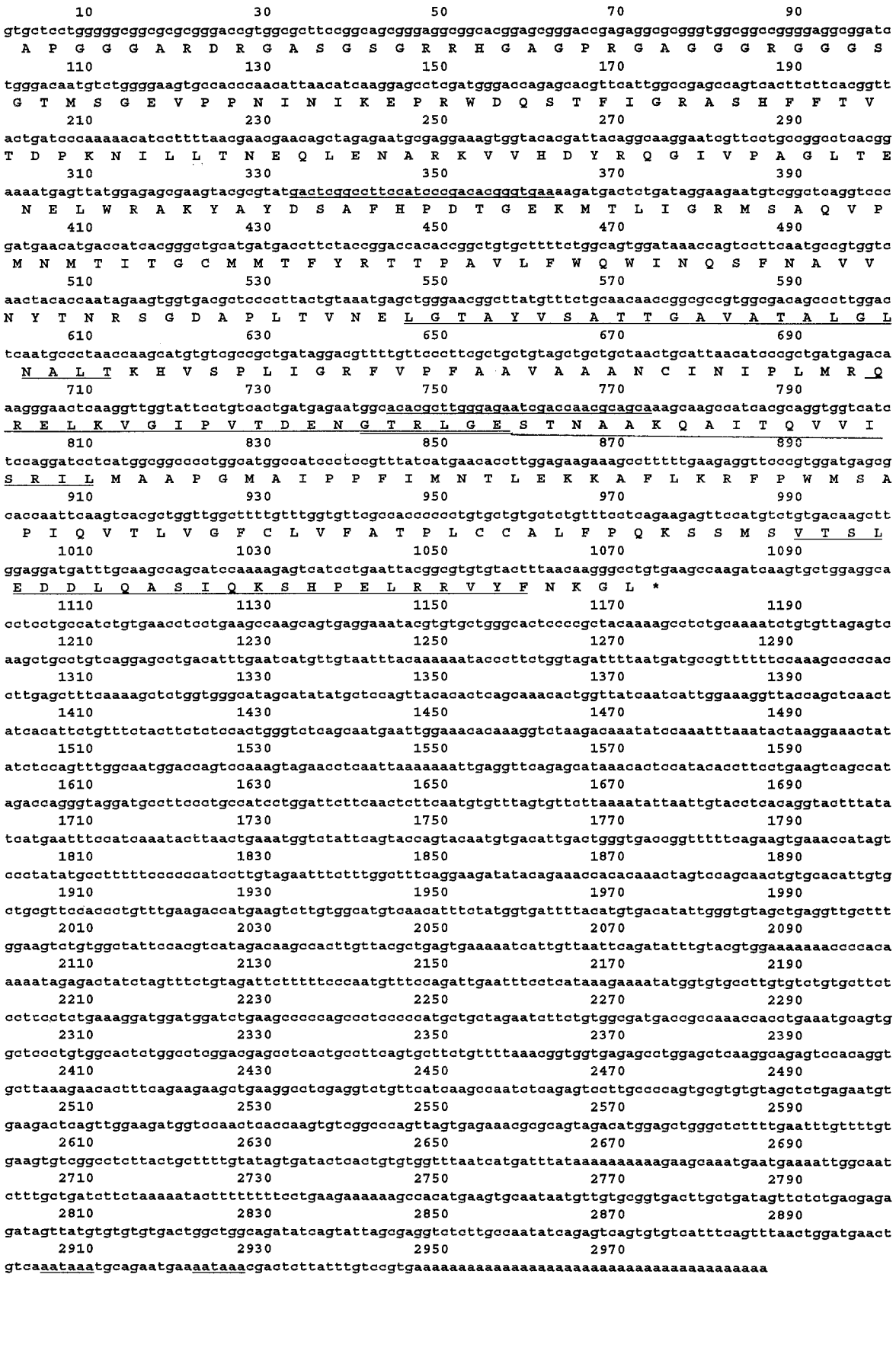


Fig. 8. Schematic representation of the complete cDNA and the obtained clones. The coding sequence is depicted as a gray box. N, B, and H indicate the positions of the restriction sites for NarI, BamHI, and HindIII, respectively. ccr1 and ccr2 denote the two oligonucleotides (30-mers) used for sequencing and screening the rat liver cDNA library. 16, 164, and 164-16 denote the different cDNA clones obtained by screening of the cDNA library (from Glerum *et al.*, 1993)

Fig. 9. Complete cDNA sequence of clone 164-16. The protein sequence translated from the open reading frame is shown in the one-letter code. The stop codon at position 1073 is denoted by an asterisk. The underlined amino acid sequences represent the obtained internal peptide sequences (cf. Table II). The first and second underlined DNA sequences correspond to the ccr2 and ccr1 probes, respectively. Two polyadenylation signals (in boxes) are found at positions 2905 and 2922. The first in frame ATG codon appears at position 108 (from Glerum *et al.*, 1993)



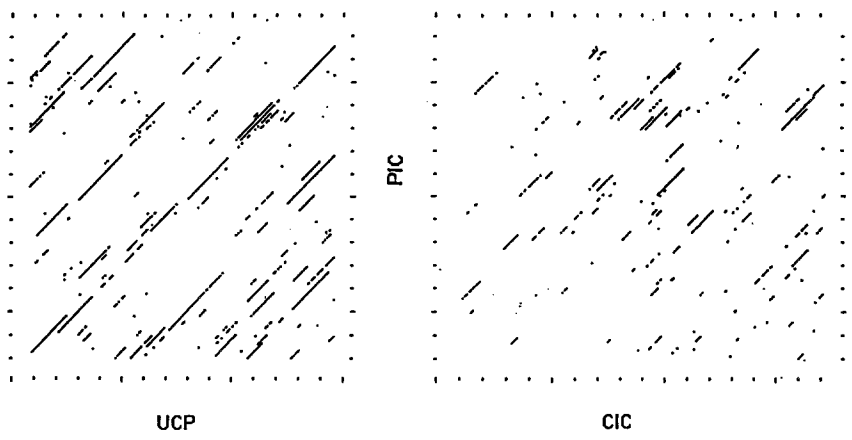


Fig. 10. Comparison of the rat liver tricarboxylate (CIC) and of the rate uncoupling protein (UCP) carrier protein sequences with the phosphate carrier (PIC) sequence. The calculations were made with the program DotPlot of the GCG Sequence Analysis Package (version 6.1, 1989), applying a window of 30 and stringency of 12 (from Glerum *et al.*, 1993).

single open reading frame encoding the tricarboxylate carrier and 1914 bp comprising the 3′-non-coding region. The cDNA is terminated by a poly(A) tail, which is preceded by a consensus polyadenylation signal sequence (30) 25 bp upstream. A second such sequence occurs a further 17 bp upstream. We have assigned the translation initiation site to an in-frame ATG codon 108 bp downstream of the start of the cDNA. This is the only methionine in the 5′ region of the clone. A purine, specifically an A, is found three nucleotides upstream of this codon, in fact considered important for initiation of translation (Kozak, 1986).

This cDNA thus codes for a 322 amino acid protein, with a predicted molecular weight of 35,546. The deduced protein sequence displays no homology to the other mitochondrial carriers cloned thus far (Walker *et al.*, 1987; Ferreira *et al.*, 1989; Ridley *et al.*, 1986). As shown in Fig. 10, a sequence comparison of the tricarboxylate carrier (CIC) with the phosphate carrier (PIC), using a window of 30 amino acids and a stringency of 12, reveals no stretches of homology between the two. In contrast, a similar comparison between the PIC and the UCP reveals the homology between these two proteins. The tripartite structure, which is a feature of these two proteins, also results in internal homology. The tricarboxylate carrier, when compared to itself, does not reveal any internal homology and does not suggest the existence of a tripartite structure. This is the first example of a mitochondrial carrier which does not fit into the putative gene family. A hydropathy plot (Fig. 11) of the carrier protein suggests that the carrier may have 5 or 6 membrane-spanning regions, with a highly hydrophobic C-terminus and a relatively hydrophilic

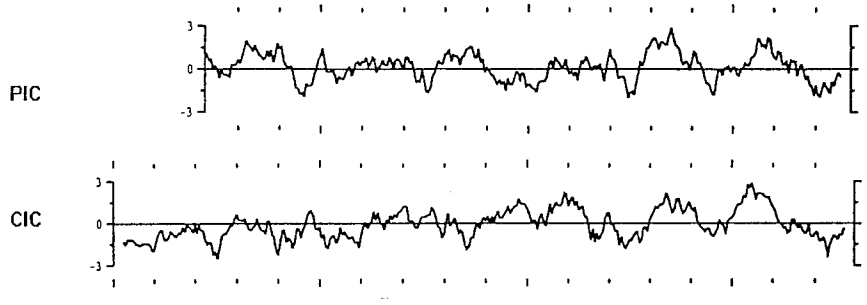


Fig. 11. Hydropathy profiles of the rat liver tricarboxylate (CIC) and phosphate carrier (PIC). A window of 9 was used in the PepPlot program of the GCG Sequence Analysis Package (version 6.1, 1989).

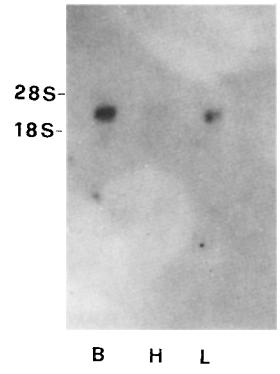


Fig. 12. Northern analysis of brain, heart, and liver using a liver-specific probe. Total cellular RNA from rat tissues was hybridized with a probe consisting of the nucleotides 418–1095 of clone 164-16. The tissues brain, heart, and liver are denoted B, H, and L, respectively (from Glerum *et al.*, 1993).

N-terminal region. Figure 11 also shows the profile generated by a similar analysis of the phosphate carrier, in which the six transmembrane regions are more clearly visible.

EXPRESSION OF THE TRICARBOXYLATE CARRIER IN DIFFERENT TISSUES

Whole cell RNA preparations from rat liver, brain, and heart were also screened by Northern analysis to assess the expression of this protein in various tissues. As shown in Fig. 12, a 677-bp EcoRI-HindIII fragment from the coding region of clone 164 was used to probe the Northern blot, where it hybridized with mRNA from brain and liver. The probe failed to bind mRNA from heart, which is compatible with the lack of carrier activity found in heart mitochondria. From the Northern analysis, a size of approximately 3 kb was determined for the tricarboxylate carrier mRNA.

CONCLUSIONS

The preparation of the tricarboxylate carrier we have discussed above has the following features. It can be purified from intact mitochondria or from mitoplasts. Reconstituted in liposomes, the preparation catalyzes citrate transport with substrate and inhibitor specificities close to the mitochondrial activities. The protein has a molecular mass of 38 kDa and is substantially different from lower-molecular-mass proteins obtained in other laboratories. The latter cannot be considered proteolytic degradation products of the 38-kDa protein. From protein sequence information, a full-length cDNA clone has been obtained. The protein reveals no internal tripartite structure or homology with the family of mitochondrial anion transporters, sequenced until now.

NOTE ADDED IN PROOF

After the submission of this article, a study had been published (Kaplan, R. S., Mayor, J. A., and Wood, D. O. (1993) *J. Biol. Chem.* **268,** 13682–13690) in which another mitochondrial tricarboxylate transport protein had been sequenced. This protein appears to be analogous to other mitochondrial carriers and does not have similarities with the sequence described in this study. This confirms our suggestion that the two preparations are dissimilar and supports our proposal of the existence of two distinct citrate transport proteins in mitochondria.

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