Characterization of a second member of the subfamily of calcium-binding mitochondrial carriers expressed in human non-excitabile tissues

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We have recently identified a subfamily of mitochondrial carriers that bind calcium, and cloned *ARALAR1*, a member of this subfamily expressed in human muscle and brain. We have now cloned a second human *ARALAR* gene (*ARALAR2*) coding for a protein 78.3 % identical to Aralar1, but expressed in liver and non-excitabile tissues. Aralar2 is identical to citrin, the product of the gene mutated in type-II citrullinaemia [Kobayashi, Sinasac, Iijima, Boright, Begum, Lee, Yasuda, Ikeda, Hirano, Terazono et al. (1999) Nat. Genet. 22, 159–163]. A related protein, DmAralar, 69 % identical to Aralar1, was found in *Drosophila melanogaster*, the *DMARALAR* locus lying on the right arm of the third chromosome, band 99F. The N-terminal half of Aralar2/citrin is able to bind calcium and this requires the

presence of the two most distal EF-hands. The localization of Aralar2/citrin expressed in human cell lines is mitochondrial, the C-terminal half containing sufficient information for import and assembly into mitochondria. The C-terminal half of Aralar proteins is related to the yeast *YPR020c* gene, with a very high sequence conservation (54.3 % identity), suggesting that these proteins play an important role. Thus Aralar proteins are probably expressed in all tissues in an isoform-specific fashion, where they function as calcium-regulated metabolite (possibly anionic) carriers.

Key words: chromosomal localization, citrullinaemia, *Drosophila*, EF hands, tissue-specific expression.

INTRODUCTION

Metabolite transport across the inner mitochondrial membrane is mediated by a large superfamily of mitochondrial carrier (MC) proteins, including about 35 members in *Saccharomyces cerevisiae* [1]. These proteins are characterized by the presence of three repeated regions, each about 100 amino acids long, which contain a conserved sequence motif [2,3], and two putative transmembrane domains (TMs). There is growing evidence that deficiencies in a number of MCs are associated with human disease, particularly mitochondrial myopathy [4,5]. However, the function of many of these proteins is still unknown [6].

We have recently identified a subfamily of MCs that are calcium-binding mitochondrial carriers (CaMCs), and cloned *ARALAR* (*ARALAR1*), a member of this subfamily expressed in human muscle, heart and brain [7]. CaMC subfamily members have a bipartite structure: the N-terminal half harbours four EF-hand domains whereas the C-terminal half has the characteristic features of the mitochondrial solute-carrier superfamily. The presence of calcium-binding domains in Aralar1 and subfamily members opens up the possibility that these proteins might be involved in calcium-regulated metabolite transport in mitochondria and/or participate in calcium-modulated mitochondrial functions. The identity of mitochondrial calcium transporters is still unknown, despite efforts to purify these proteins [8,9], and the calcium sensor for the permeability transition pore also awaits identification. In view of the possible importance of Aralar1 subfamily members in calcium sensing by mitochondria, it was surprising that *ARALAR1* expression was restricted to the excitable tissues. Therefore, we have searched for members of this subfamily in human tissues that lacked *ARALAR1* expression. In the present article, we report the cloning and

characterization of an *ARALAR*-related gene, *ARALAR2*, expressed in human non-excitabile tissues. In addition, we have also found a member of this subfamily in *Drosophila melanogaster*. Interestingly, *ARALAR2* has now been found to be mutated in adult-onset type-II citrullinaemia [10].

METHODS

Isolation of *ARALAR*-related sequences

Full-length protein and nucleotide sequences of human and mouse *ARALAR* genes were used to search the National Center for Biotechnology Information (NCBI, Bethesda, MD, U.S.A.) expressed sequence tag (EST) and genomic database. Several overlapping human EST clones in GenBank (accession numbers AA410516, AA99865, AA511635, T55265, AA360112 and H59215) and human BAC (bacterial artificial chromosome) clones (AC002450 and AC002540) were found which showed high degrees of similarity with *ARALAR1*. An aliquot of human liver cDNA library (Quick-clone cDNA, Clontech) was PCR-amplified by primers T554 (5'-GCAGATTTATATGAGCCAA-GG-3') and T556 (5'-AGAAATGACGTCATGGGTG-3'), designed according to human BAC clone AC002540 and human EST clone H59215 respectively. A PCR product was obtained homologous to the 3' region of *ARALAR1*. PCR amplification by primers T550 (5'-AGTCAGTGGGTCCCGCAGT-3'), designed from human EST AA199865, homologous to the 5' end of *ARALAR1*, and T555 (5'-CCTTGGGCTCATATAAATCT-GC-3'), complementary to T554, was also carried out. Both PCR products, of 1233 and 885 bp, respectively, were fused by partial *SpyI* digestions, obtaining a 2119-bp fragment containing the full-length coding region, and subcloned into pUC8 to produce

Abbreviations used: MC, mitochondrial carrier; TM, transmembrane domain; IPTG, isopropyl β -D-thiogalactoside; CTLN2, adult-onset type-II citrullinaemia; CaMC, calcium-binding mitochondrial carrier; S-CaMC, short CaMC; L-CaMC, long CaMC; ADT, adenine nucleotide translocator; EST, expressed sequence tag; DAPI, 4',6-diamino-2-phenylindole; FISH, fluorescence *in situ* hybridization; BAC, bacterial artificial chromosome.

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pUC-*ARALAR2*. Double-stranded nucleotide sequencing was performed by the Sanger dideoxy chain-termination method, using the Sequenase system (USB, Cleveland, OH, U.S.A.), and [α - 35 S]dATP.

The *D. melanogaster* EST AA696927 derived from ovary was purchased from Genome Systems (St. Louis, MO, U.S.A.). The sequencing strategy involved both sequencing subcloned restriction fragments with universal primers and 20-mer oligonucleotides (Isogen Bioscience BV, Maarssen, The Netherlands) to fill remaining gaps. DNA sequences were analysed using the GCG Wisconsin package.

Expression of N-terminal fragments of Aralar2/citrin and $^{45}\text{Ca}^{2+}$ -overlay assays

To express the N-terminal regions of Aralar2/citrin, amino acids 9–278 and 37–278, two different DNA fragments were obtained by PCR and ligated into the *Escherichia coli* expression vector pQE32 (Qiagen) at the appropriate restriction sites. We used the oligonucleotides MalT55 (5'-GGAATTCAAGAGAGCAGATCCAGC-3', nucleotides 64–84 including an *EcoRI* site) and T555 (5'-CCTTGGCTCATATAAATCTGC-3', complementary to 886–866) to subclone amino acids 9–278 of Aralar2/citrin (His₆-Aralar2) into pBluescript KS. The insert was then obtained by digestion with *Bam*HI/*Hind*III and ligated into pQE32 (His₆-Aralar2). Oligonucleotides T557 (5'-CCCAATGACTTTGTCACG-3', nucleotides 149–168) and T555 for expression of amino acids 37–278 (His₆- Δ Aralar2) were used.

E. coli strain M15 was transformed with either pQE32-Aralar_{9–278} or pQE32-Aralar_{37–278}, and grown at 37 °C until $A_{600} = 0.4$ – 0.6 . Expression of recombinant proteins was then induced with 2 mM IPTG (isopropyl β -D-thiogalactoside) and growth was continued for 4 h. Cells were harvested by centrifugation and the pellets were lysed in 400 μ l of 2 $\times$ Laemmli solution (125 mM Tris/HCl, pH 6.8, 20% glycerol, 4% SDS, 15 mM EDTA and 10% β -mercaptoethanol). Aliquots (20 μ l) from samples before and after induction with IPTG were analysed by SDS/PAGE performed on a 10% gel. After electrophoresis, the proteins were transferred on to a nitrocellulose membrane at a constant current of 100 mA for 1 h in Tris/glycine buffer. $^{45}\text{Ca}^{2+}$ -overlay was performed essentially as described in [11]. After transfer, the membrane was washed in IMK buffer (10 mM imidazole-HCl, pH 6.8, 5 mM MgCl_2 and 60 mM KCl) for 1 h with three changes of buffer. The membrane was then incubated in the same buffer with 5 $\mu\text{Ci/ml}$ $^{45}\text{Ca}^{2+}$ for 10 min. After incubation, the membrane was rinsed with 40% ethanol for 5 min, three times. The membrane was dried and it was exposed to film for 1 day at -80 °C.

Northern-blot analysis

A human multiple-tissue Northern blot containing 2 μg of poly(A)⁺ RNA per lane (Clontech) was used as described earlier for *ARALAR1* [7]. Total RNA was isolated from human cell lines by standard methods. Total RNA (20 μg) was electrophoresed on 1% formaldehyde/agarose gels and transferred on to nylon membranes. For expression analysis of *ARALAR2*, to avoid cross-hybridization between Aralar1 and Aralar2 transcripts, we chose a probe corresponding to most of the N-terminal coding sequence of *ARALAR2* (nucleotides 149–885, coding for amino acids 37–271), since Aralar1 and Aralar2 are less conserved in this half than in the C-terminal half. The corresponding 0.73-kb [α - ^{32}P]dCTP-labelled probe was used for Northern-blot analysis [7]. Membranes were hybridized and washed under high-stringency conditions as described earlier [7]. The blot was

stripped in 0.1% SDS at 100 °C for 30 min and reprobed under identical conditions with a rat β -actin probe [7].

Expression constructs for eukaryotic cells

An eight-amino-acid FLAG-epitope tag (peptide DYKDDD-DK) was introduced at the C-terminus immediately preceding the termination codon of Aralar2/citrin as follows; two complementary oligonucleotides containing the FLAG sequence and the stop-codon signal (coding strand, FLAG1, 5'-GACT-ACAAGGACGACGATGACAAGTGAG-3'; and non-coding strand, FLAG2, 5'-AATTCTCACTTGTTCATCGTCCTTGTA-GTC-3'), were annealed, generating a 32-bp 5'-blunt ended and 3'-*EcoRI*-digested fragment. They were then ligated with a 0.56-kb *Pst*I/blunt-end fragment, generated by PCR using the oligonucleotides T558 (5'-TGGGCTCCACCAATAGC-3'; complementary to nucleotides 2065–2048 of *ARALAR2*) and the internal primer T55-5 (5'-GTGAGGGATAAATTTATG-3'; nucleotides 1285–1303), and by posterior *Pst*I digestion. Finally, both *Pst*I/blunt-end and blunt-end/*EcoRI* fragments were subcloned into pUC8 by *Pst*I/*EcoRI* double digestion. The expression vector carrying the 2.1-kb complete coding sequence of FLAG-tagged Aralar2/citrin was constructed by joining the *Bam*HI/*Pst*I DNA fragment containing the 3' end of pUC-*ARALAR2* with the C-terminal *Pst*I/*Bam*HI 0.56-kb fragment of *ARALAR2* fused to the FLAG epitope into the bicistronic expression vector pIRES1hyg (Clontech) after digestion with *Bam*HI. The resultant vector was designated pIRES-*ARALAR2*.

To obtain the truncated Aralar2/citrin (amino acids 306–675, CTAralar2-FLAG), a DNA fragment was generated by PCR with the following primers: 5'-ACCATGGCTGAGGCCAGAGGCA-3' [nucleotides 953–971, containing a new initiation codon (underlined)] and oligonucleotide FLAG2, using as template pIRES-*ARALAR2*. The resulting PCR fragment was subcloned into the pIRES1hyg vector, generating pIRES-CTAralar2-FLAG. All constructs were confirmed by sequencing.

Cell culture and transfection of HEK-293T cells

HEK-293T cells were cultured in Dulbecco's modified Eagle's medium containing 5% foetal bovine serum (Gibco-BRL) at 37 °C in a 7% CO₂ atmosphere. Cells were grown in 6-cm dishes and transiently transfected using the calcium phosphate coprecipitation method as described in [7]. Stable cell lines expressing Aralar2-FLAG were selected for hygromycin resistance by plating for 2 weeks in media containing 400 $\mu\text{g/ml}$ hygromycin B (Calbiochem) and maintained at 200 $\mu\text{g/ml}$. Clonal lines were isolated and verified by immunofluorescence and Western-blot analysis.

Immunofluorescence analysis and mitochondria-specific staining

For the localization of FLAG-tagged Aralar2/citrin, living cells were incubated with MitoTracker Red CMXRos (Molecular Probes) to identify mitochondria and with anti-FLAG M2 antibodies as described earlier [7].

Fluorescence *in situ* hybridization (FISH) to polytene chromosomes

Larval polytene chromosomes from wild-type *D. melanogaster*, strain Oregon R, were prepared as described previously [12]. *DMARALAR* clone was labelled with biotin using Biotin-Nick Translation Mix (Boehringer Mannheim). After hybridization [12] the probe was detected with Fluorlink CY3-avidin

(Amersham) and the chromosomes were counterstained with 4',6-diamino-2-phenylindole (DAPI). Slides were analysed using a Zeiss Axioplan epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera (Photometrics). The fluorescent signals from the CY3 (red) and DAPI (blue) staining were recorded separately as grey-scale digital images and then pseudocoloured and merged using Adobe Photoshop.

RESULTS

Cloning and structural analysis of *ARALAR2*

To identify human cDNAs related to *ARALAR1*, we used the sequences of *ARALAR1* and its *Caenorhabditis elegans* orthologue (U00052) and the FASTA [13] and BLAST algorithms [14]. We detected a set of related human ESTs and genomic

BACs that showed high homology to 5' and 3' regions of the *ARALAR1* gene (with a degree of identity at the nucleotide level of over 70–80%), in agreement with the existence of a novel isoform of *ARALAR* that was designated *ARALAR2*. Two overlapping fragments containing the full-length coding sequence of *ARALAR2* were obtained by PCR amplification of human liver cDNA using primers based on human ESTs and BAC human clones, and sequenced. The partial cDNA of *ARALAR2* is 2119 bp long and encodes a protein of 675 amino acids that was 78.3% identical to Aralar1. The translation-initiation site was assigned to nucleotides 41–43. This region (ATC⁴¹ATGG) fits well with the consensus pattern for eukaryotic translation initiation, with only one mismatch at the –2 position (T instead of C) [15]. The predicted protein has a calculated molecular mass of 74 kDa and a theoretical isoelectric point of 9.3. Like Aralar1,

Figure 1 Comparison between Aralar-subfamily proteins and chromosomal localization of *DMARALAR*

(A) Alignment of human Aralar1 and 2 (Aralar2/citrin) and the *D. melanogaster* orthologue DmAralar. Dashes indicate amino acid identity and dots indicate gaps. The locations of the six potential TM segments are indicated by horizontal lines. The putative EF-hand domains are indicated by shaded boxes. The proteins were aligned using the Pileup program from the GCG Wisconsin package. Accession numbers for *ARALAR2* and *DMARALAR* are Y17571 and Y18197, respectively. (B) Comparison of human Aralar 1 and 2 (Aralar2/citrin), DmAralar and *C. elegans* Q21153. At the top is a schematic representation of the structure of Aralar proteins. The percentage identities and similarities between Aralar-subfamily proteins for each characteristic half are shown at the bottom. The proteins were compared using the Bestfit program. (C) Chromosomal localization of the *DMARALAR* gene. Main image: general view of a *D. melanogaster* polytene chromosome (blue) showing a single hybridization signal (red) on the right arm of the third chromosome (3R). Inset: close view of the 3R arm. *DMARALAR* (red) hybridized at polytene chromosome band 99F.

Figure 2 Northern-blot analysis for *ARALAR2* expression in human tissues and cell lines

(A) Tissue distribution of human *ARALAR2* expression. A 32 P-labelled *ARALAR2* partial cDNA probe was used. The molecular-mass markers are shown in kb (upper panel). The blot was stripped subsequently and reprobed under identical conditions with a rat β -actin control probe (lower panel). (B) *ARALAR2* expression in monkey COS cells and human cell lines. Total RNA (20 μ g) obtained from different cells [human neuroblastoma (SK-N-MC and SH-SY-5Y), human astrocytoma (U87-MG), African green monkey kidney COS cells, human kidney (HEK-293), human hepatoma (Hep-G2), human T-lymphocytes and a human lymphoblastoma cell line], were processed by standard methods and hybridized with the probe mentioned in the Methods section. Staining with ethidium bromide was realized to verify the amount of RNA loaded on the gel (lower panel). A single transcript of around 3.1 kb is evident in both human tissues and human and monkey cell lines.

this protein displays two different regions; a C-terminal half of 369 amino acids with a pI of 10.42 and characteristics of the MC superfamily [2], and an N-terminal acidic extension of 306 amino acids, with a pI of 5.69 and a number of EF-hand motifs. Putative EF hands and MC motifs were predicted by the Blocks Search Program; the positions of these domains exactly matched those in Aralar1 (see Figure 1A).

The *ARALAR2* cDNA sequence matched a collection of approximately 30 human, mouse and rat ESTs. As for *ARALAR1*, the mouse ESTs (AA511635, W76821 and W77458) showed a high degree of similarity to human *ARALAR2* at both the nucleotide (89%) and amino acid levels (96%). A partial *ARALAR2* cDNA, nucleotides 646–2119, matched those of the human genomic BAC clones, AC002450 and AC002540. These BAC clones have been located on chromosome 7 at q21.1–q22; therefore this is the *ARALAR2* locus. Kobayashi et al. [10] have now reported that mutations in the gene coding for Aralar2 cause adult-onset type-II citrullinaemia (CTLN2). These authors have used positional cloning to localize the CTLN2 gene (*SCL25A13*) to chromosome 7q21.3, and the encoded protein has been named citrin. Its amino acid sequence matches that of Aralar2, which we will denote Aralar2/citrin.

Tissue distribution of *ARALAR2* differs from that of *ARALAR1*

Northern-blot analysis of RNA derived from a series of human tissues (Figure 2A) revealed that the mRNA for Aralar2 was expressed at its highest levels in the liver, a tissue where Aralar1 mRNA was not detectable [7], with lower levels in kidney, pancreas, placenta, heart and brain. Similar results were obtained by Kobayashi et al. [10]. This tissue-distribution pattern is entirely different from that reported previously for Aralar1 mRNA (two transcripts of 2.9 and 3.2 kb) that had their highest

levels in heart and skeletal muscle [7]. Therefore, it appears that most, if not all, tissues express *ARALAR* genes, *ARALAR2* being expressed in non-excitable tissues, and *ARALAR1* in the excitable ones.

The *ARALAR2* transcript was also detected in several human cell lines of different origins (Figure 2B), in agreement with the generalized expression of *ARALAR2*. Moreover, hybridization signals of about the same intensity were observed in the different human cell lines tested, with the exception of HepG2, a human cell line derived from liver, which showed a slightly more intense hybridization band (Figure 2B), in agreement with its tissue-specific expression pattern. The transcript size was identical, approximately 3.1 kb, in cell lines and human tissues.

Cloning of *Drosophila* *ARALAR*-related gene

To identify *Drosophila* cDNAs related to *ARALAR*, we used *ARALAR1* sequence and the BLAST algorithm [14] to probe the dbEST database. We found three overlapping *D. melanogaster* EST clones (AA696927, AA951820 and AA801718), coding for the N-terminal half of a protein orthologous with respect to Aralar, which we designated DmAralar. Clone AA696927 has been completely sequenced and encodes a full-length *DMARALAR* cDNA of 2748 bp, with a 117-bp 5'-untranslated region, a 2046-bp open reading frame and a 585-bp 3'-untranslated region. The start-codon (18 ATG)-flanking sequence (GGAATGCC) is not frequently observed [15] but has an in-frame stop codon 69 bp upstream of it. The 3'-untranslated region is polyadenylated 17 nucleotides downstream of the polyadenylation signal (2708 AATAAA). *DMARALAR* cDNA encodes a protein of 682 amino acids, which shares strong similarity with Aralar1 and Aralar2/citrin, 69 and 66% respectively (Figure 1A). In *Drosophila*, as in *C. elegans* [7], a single

Figure 3 Calcium-binding domains in Aralar-subfamily proteins

(A) Alignment of putative EF-hand domains in Aralar-subfamily members. The amino acids that contribute to the octahedral Ca^{2+} co-ordination cage are labelled x, y, z, -x, -y and -z. The amino acid residues that do not satisfy the co-ordination bond of canonical EF hands are in italics and underlined. (B) $^{45}\text{Ca}^{2+}$ ligand-blotting analysis of the N-terminal half of Aralar2/citrin. A schematic diagram of the primary structures of His₆-Aralar2 and His₆-ΔAralar2 is shown, where the EF-hand domains are indicated by black boxes. Aliquots of *E. coli* lysates expressing His₆-Aralar2 before (lanes A) and after IPTG induction (lanes B) and IPTG-induced His₆-ΔAralar2 (lanes C) were separated by electrophoresis (Coomassie Brilliant Blue staining, left-hand panel), blotted on to nitrocellulose membranes and assayed for $^{45}\text{Ca}^{2+}$ binding. An autoradiograph of the proteins labelled with $^{45}\text{Ca}^{2+}$ is shown (right-hand panel). After exposure of the membrane, a single signal of approximately 35 kDa corresponding to His₆-Aralar2 was detected.

type of related ESTs homologous to human *ARALAR* sequences has been detected. Attempts to find other isoforms have been unsuccessful.

Figure 1(A) shows that the sequence dissimilarities between human and *Drosophila* Aralar isoforms are concentrated in the N-terminal acidic half, in the region between putative TM helices 4 and 5, and at the C-terminal end. Human Aralar proteins (Aralar2/citrin and Aralar1) and DmAralar displayed strong similarity, 62% and 65% respectively, to the *C. elegans* orthologue Q21153 [7]. The similarity encompassed the total amino acid sequence of these proteins, but it was higher within the C-terminal half, which contains the MC consensus sequences (Figure 1B).

FISH of *DMARALAR* probes to *D. melanogaster* polytene chromosomes revealed that the *DMARALAR* locus lies on the right arm of the third chromosome (3R), band 99F (Figure 1C).

Calcium-binding properties of Aralar2/citrin and DmAralar

The acidic N-terminal half of the Aralar-subfamily members shows similarity with other EF-hand calcium-binding proteins, especially with calmodulin and related proteins (25–30% identity). Figure 3(A) shows an alignment of the EF-hand domains in Aralar1, Aralar2/citrin, Q21153 and DmAralar. All four proteins contain four EF-hand domains at conserved positions. However, whereas the first and second EF hands are potentially capable of binding calcium in Aralar1, Aralar2/citrin and DmAralar (see Figure 3A), neither the third (Aralar1, Aralar2/citrin and DmAralar) or fourth (Aralar2/citrin and DmAralar) are *bona fide* EF-hands [16,17] (Figure 3A). On the other hand, in *C. elegans* Q21153, the first EF-hand is non-canonical (Figure 3A).

To examine the possibility that the N-terminal half of Aralar2/citrin could bind calcium, we expressed two His₆-tagged truncated forms of Aralar2/citrin (His₆-Aralar2, His₆-ΔAralar2) in *E. coli*,

and tested their ability to bind $^{45}\text{Ca}^{2+}$ in an overlay assay. His₆-Aralar2 contains most of the N-terminal half of Aralar2/citrin (amino acids 9–278), including all four EF-hand domains, whereas His₆-ΔAralar2 (amino acids 37–278) lacks most of the first EF hand (9 out of 12 amino acids). As shown in Figure 3(B) (left-hand panel), one major protein band of around 30–35 kDa, corresponding to the predicted molecular mass of His₆-Aralar2 or His₆-ΔAralar2, appears in lysates of *E. coli* after induction with 2 mM IPTG (lanes B and C) and is not present without IPTG induction (lane A). $^{45}\text{Ca}^{2+}$ -overlay experiments shown in Figure 3(B) (right-hand panel) indicate that His₆-Aralar2 is labelled clearly, whereas His₆-ΔAralar2 is not. These results indicate that by eliminating the first EF-hand domain of Aralar2/citrin, the protein loses its calcium-binding capacity. These results support the notion that neither the third nor fourth EF-hands bind calcium and that calcium binding by EF-hands 1 and 2 is prevented by eliminating one of these EF-hands, as observed by other calcium-binding proteins [18].

Subcellular localization of Aralar2/citrin in mitochondria

To assess the mitochondrial localization of Aralar2/citrin, we expressed FLAG-tagged Aralar2/citrin (the FLAG epitope at the C-terminal end of the protein) in HEK-293T cells. Immunostaining with anti-FLAG antibodies and visualization by fluorescence microscopy was carried out 24 h after transient transfection or in stably transfected cells. Immunofluorescence with anti-FLAG antibodies revealed a granular cytoplasmic staining and the typical morphology of mitochondria (Figure 4A). The presence of Aralar2/citrin epitopes in mitochondria was confirmed by co-localization of the anti-FLAG positive cytoplasmic organelles with that of MitoTracker Red CMXRos, a mitochondrial-specific fluorescent vital dye [19] (Figure 4B).

As for other members of the MC family [20], Aralar2/citrin has no obvious potential cleavable presequence (Figure 1A).

Figure 4 Localization of Aralar2-FLAG and CTAralar2-FLAG proteins in mitochondria

HEK-293T cells expressing *ARALAR2* (**A** and **B**, stable cell lines transfected with pIRES-Aralar2-FLAG; **C** and **D**, transient transfections with pIRES-CTAralar2-FLAG) were incubated with MitoTracker, fixed, permeabilized, and incubated with anti-FLAG and FITC-conjugated secondary antibodies. Identical fields stained for MitoTracker (**B**, **D**) and anti-FLAG (**A**, **C**), visualized by fluorescence microscopy, are presented.

However, since Aralar-subfamily members are clearly different from other MCs, we tested whether the mitochondrial targeting signals of Aralar2/citrin were present exclusively in the C-terminal half of the mature protein and not at the N-terminal half. To this end, HEK-293T cells were transiently transfected with a truncated and FLAG-tagged form of Aralar2/citrin (CTAralar2-FLAG) that contained the complete MC homology sequence (amino acids 306–675) and lacked its N-terminal half. As shown in Figures 4(C) and 4(D) the staining patterns of CTAralar2-FLAG (green) and MitoTracker (red) were exactly the same, indicating that the C-terminal half of Aralar2/citrin contained sufficient information for import and assembly into mitochondria.

Relationship between Aralar-subfamily members and other mitochondrial carriers

Nelson et al. [6] have identified and aligned the 35 MCs from yeast and have studied their relatedness. The MC sequence of Aralar-subfamily members is similar to that of Q12482, the protein coded by the yeast gene *YPR020c*: there is 54.3% identity between the C-terminal half of Aralar1 (amino acids 323–602) or Aralar2/citrin (amino acids 325–604) and Q12482 (amino acids 521–811). Q12482 protein has 902 amino acids and its long (approximately 500 amino acids) N-terminal extension has no homology with Aralar1 or with anything in GenBank [6]. The strong conservation of the MC sequence in Aralar-subfamily members and yeast Q12482 is a possible indication that the transported species is common to all. Thus the similarity between Aralar1 and Q12482 matches that of isoforms of the adenine nucleotide translocator (ADT) in human and yeast (identity of human versus yeast ADT2 is 54.6%).

The MCs known so far are transporters of charged or polar substrates. It is believed that some of the charges that occur in TM segments interact with the charged transported species.

Figure 5 Charge distribution in Aralar1 and Aralar2/citrin

(**A**) Representation of the C-terminal half of Aralar1 and Aralar2/citrin according to its homology with yeast ADT2 [34]. Charged amino acids (diamonds, glutamate or aspartate; squares, arginine or lysine) and conserved prolines (circles) in potential TM domains are shown. Amino acid numbers correspond to Aralar1 sequence. Changes in charged amino acids in Aralar2/citrin versus Aralar1 are indicated (A2). (**B**) Comparison of total charges of all or even-numbered TM segments of yeast ADT2, yeast tricarboxylate carrier (TXTP1), rat carnitine/acyl carnitine exchanger [3] and yeast ornithine carrier, ARG11 [21]. The positions of TM segments from yeast proteins were taken from [6] and those of the rat carnitine/acyl carnitine carrier from [3].

The TM segments are hydrophobic or amphiphilic, most of the charges appearing to fall on one side of a helical-wheel plot [6]. This information has been used to predict which surface of the helices should face the translocation pathway, especially for the even-numbered helices that contain most of the charge [6]. The total charge in TM segments of Aralar1 and Aralar2/citrin is 4+ (Figure 5). Figure 5(B) shows the charges in TM segments of a number of MCs. Based on the global charge of TM segments it would appear that Aralar proteins are anion carriers.

DISCUSSION

The subfamily of MCs with EF hands (CaMC) is made up of two groups of proteins that differ in the sequence of the C-terminal half, i.e. that containing the MC homology sequence (Table 1). These two groups are related to the products of yeast genes *YNL083w* [22] and *YPR020c*, and we have designated them S-CaMC (for short) or L-CaMC (for long), according to the length of their polypeptides. The genome of *C. elegans* contains at least three CaMC genes [7]. Two of these genes (*CEF17E5* and *CEF55A11*) code for putative proteins Q19529 and Q20799 (TREMBL database) that belong to the S-CaMC group and are extremely similar to each other (70% identity within the C-terminal region). These two proteins are very similar to the

Table 1 The two groups of genes of the MC subfamily with EF hands

The sequence of *YNL083w* is different in two yeast backgrounds. The length of Ynl083p [545 amino acids, (aa)] corresponds to the longest of the two [22,35]. EF, no EF, refer to the presence or absence of EF-hand domains in the proteins.

Organism	Short CaMC		Long CaMC	
	Gene	Protein	Gene	Protein
<i>S. cerevisiae</i>	<i>YNL083w</i>	Ynl083p, 545 aa/EF	<i>YPR020c</i>	Q12482, 902 aa/no EF
<i>C. elegans</i>	<i>CEF17E5</i>	Q19529, 531 aa/EF	<i>CE01348</i>	Q21153, 702 aa/EF
	<i>CEF55A11</i>	Q20799, 588 aa/EF		
<i>D. melanogaster</i>			<i>DMARALAR</i>	DmAralar, 682 aa/EF
Rabbit	<i>AF004161</i>	Efinal, 475 aa/EF		
Human			<i>HSARALAR1/SLC25A12</i>	Aralar1, 678 aa/EF
			<i>HSARALAR2/SLC25A13</i>	Aralar2/citrin, 675 aa/EF

rabbit protein Efinal [23] (55 % identity within the C-terminus). Efinal, Q19529 and Q20799 are highly homologous to protein Ynl083p encoded by yeast gene *YNL083W* (33–35 % identity within the C-terminus).

The L-CaMC group has only one member in *C. elegans*, Q21153, which is homologous to Aralar1, Aralar2/citrin, DmAralar and, as noted by Nelson et al. [6], to protein Q12482, encoded by yeast gene *YPR020c*, the longest of all yeast proteins from the MC superfamily (902 amino acids). The rabbit S-CaMC Efinal has been shown to localize to peroxisomes [23], and this raises the possibility that other members of this group might also be peroxisomal. On the other hand, Aralar1 and Aralar2/citrin are mitochondrial, and it is likely that other members of the L-CaMC group share this cellular localization. Interestingly, we have found that targeting to mitochondria does not require the long N-terminal half of Aralar2/citrin, indicating that the MC homology sequence itself contains sufficient information for import of this protein, as found for a number of MCs [24] and for a presequence-deficient mitochondrial phosphate carrier [25].

We have observed that the degree of conservation of the MC homology sequence among the L-CaMC proteins is very high. There is 54.3 % identity between human and yeast L-CaMC sequences, i.e. a value higher than that of the tricarboxylate (TXTP) and phosphate (MPCP) carriers (39 % and 41.5 % identity of human and yeast TXTP1, and human and yeast MPCP, respectively) and within those obtained for the ADTs, suggesting that the function of the L-CaMC proteins is an important one. Indeed, the finding that mutations in Aralar2/citrin lead to human disease [10] is consistent with an important function for L-CaMC proteins. A comparison between the charges present in the putative TMs of L-CaMC and other mitochondrial carriers suggests that the transported molecule may be anionic. Interestingly, the yeast member of this group, Q12482, does not have EF-hand domains in its long N-terminal extension. Since *S. cerevisiae* mitochondria do not take up calcium nor react to calcium in the way that mammalian mitochondria do [26–28] this finding suggests that L-CaMC proteins may be anion carriers regulated by calcium in higher eukaryotes and by another unknown mechanism in yeast. In human Aralar2/citrin, and probably in Aralar1 and DmAralar, the two EF hands closest to the extreme N-terminus are responsible for calcium binding, and possibly calcium regulation.

For the human L-CaMCs, the present study shows that there are two Aralar proteins that are expressed in a tissue-specific manner. Other mitochondrial carriers, such as ADT or the

phosphate carrier, have isoforms that are also expressed differently in various tissues [4,5]. In the case of ADT, the different isoforms correspond to different genes, whereas the human phosphate-carrier isoforms (IIIA and IIIB) arise from alternative splicing of a single gene product [4]. The Aralar2/citrin mRNA expression pattern is comparable with that of ADT2 (and perhaps ADT3) and phosphate carrier IIIB, all three isoforms being expressed ubiquitously in non-excitable tissues. Moreover, the second isoform of these three mitochondrial carriers (ADT1, phosphate carrier IIIA and Aralar1) is expressed predominantly in heart and skeletal muscle [4,5,7]. Thus as suggested for ADT and phosphate carriers [4], Aralar2/citrin may serve for a continuous basal transport of (presumably anionic) metabolite(s) in all tissues, whereas the heart/muscle/brain Aralar1 isoform could accommodate the high metabolic turnover rate in these tissues imposed by their higher energy demand and in a calcium-regulated fashion.

CTLN2 is associated with elevations in plasma ammonia, citrulline and arginine due to a decrease in hepatic argininosuccinate synthetase levels, with normal renal argininosuccinate synthetase; mutations leading to truncation or structural changes in Aralar2/citrin have now been shown to cause this disease [10]. Kobayashi et al. [10] have argued that Aralar2/citrin may associate with members of the urea cycle, including argininosuccinate synthetase itself, and that a loss of organization due to the absence of the functional protein in CTLN2 patients might lead to destabilization or degradation of argininosuccinate synthetase. Indeed, Cheung et al. [29] and Cohen et al. [30] have provided evidence that the cytosolic and mitochondrial enzymes of the urea cycle are grouped in complexes in such a way that channeling of citrulline and ornithine out and in mitochondria is made possible [31]. A candidate protein to serve as scaffold for these complexes would thus be the citrulline/ornithine carrier. However, it has also been shown recently that the human ornithine carrier is coded by a different gene and that mutations in this gene cause hyperornithinaemia-hyperammonaemia-homocitrullinuria (HHH) [32]. Alternatively, Aralar2/citrin may be associated with the glutamate-aspartate exchanger. Mitochondrial efflux of aspartate is required as nitrogen donor in the case of urea synthesis, to produce argininosuccinate, and for the transfer of carbon (oxaloacetate) in the case of gluconeogenesis, and this process may be regulated by calcium [33]. Whether Aralar2/citrin may correspond to that exchanger or a related carrier requires further work. Therefore, the carrier/scaffold function of Aralar2/citrin together with the role of its calcium-binding domains are still open questions.

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