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Direct Evidence for Two Distinct Forms of the Flavoprotein Subunit of Human Mitochondrial Complex II (Succinate-Ubiquinone Reductase)

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Succinate-ubiquinone reductase (complex II) is an important enzyme complex in both the tricarboxylic acid cycle and aerobic respiration. A recent study showed that defects in human complex II are associated with cancers as well as mitochondrial diseases. Mutations in the four subunits of human complex II are associated with a wide spectrum of clinical presentations. Such tissue-specific clinical symptoms suggest the presence of multiple isoforms of the subunits, but subunit isoforms have not been previously reported. In the present study, we identified two distinct cDNAs for the human flavoprotein subunit (Fp) from a single individual, and demonstrated expression of these two isoforms in skeletal muscle, liver, brain, heart and kidney. Interestingly, one of the Fp isoforms was encoded as an intronless gene.

Key words: complex II, flavoprotein subunit, human mitochondria, isoforms, succinate-ubiquinone reductase.

Abbreviations: QFR, quinol-fumarate reductase; SQR, succinate-ubiquinone reductase; Fp, flavoprotein subunit; Ip, iron-sulfur protein subunit; FRD, fumarate reductase; SDH, succinate dehydrogenase.

Succinate-ubiquinone reductase (complex II or SQR) is an important enzyme complex in both the tricarboxylic acid cycle, and the aerobic respiratory chains of eukaryotic cell mitochondria and prokaryotic cells (see Ref. *1* for a recent review). A recent study showed that defects in human complex II are associated with cancers as well as mitochondrial diseases, including neurological disorders (*2*). A mutation in the flavoprotein subunit (Fp), which contains covalently bound FAD and is the catalytic site for succinate/fumarate conversion, results in a mitochondrial encephalopathy known as Leigh syndrome (*3*, *4*). Germline heterozygous mutations in the three other subunits of complex II have been shown to cause the inherited syndromes phaeochromocytoma and paraganglioma (*5*, *6*). Thus, mutations in the four subunits of human complex II are associated with a wide spectrum of clinical pathologies (*7*, *8*). Such tissue-specific clinical symptoms suggest the presence of multiple isoforms of the subunits, but subunit isoforms have not been previously identified (*9*).

Complex II catalyzes the oxidation of succinate to fumarate (succinate dehydrogenase: SDH) and transfers its reducing equivalent to ubiquinone (*1*). Complex II also catalyzes the reduction of fumarate (quinol-fumarate reductase: QFR), the reverse of the reaction catalyzed by SQR, in the respiratory chain of anaerobic bacteria and in the mitochondria of facultative anaerobic animals such as adult *Ascaris suum* (*10*). Complex II is generally composed of four polypeptides with apparent molecular masses of 70, 30, 15, and 13 kDa, and contains five prosthetic groups, i.e. one covalently linked FAD, three iron-sulfur clusters (2Fe-2S, 4Fe-4S, and 3Fe-4S), and a heme *b*. The two larger subunits, Fp and the iron-sulfur protein subunit (Ip), comprise the catalytic portion of the enzyme complex and catalyze electron transfer from succinate to artificial electron acceptors such as phenazine methosulfate (succinate dehydrogenase: SDH). The amino acid sequences of Fp and Ip are highly conserved, and their cDNAs and genes have been cloned from various species by homology probing strategies (see Ref. *1* and references therein). The presence of a two-subunit cytochrome *b* composed of large (CybL, also referred to as QPs-1 or C_{II-3}) and small (CybS, also referred to as QPs-3 or C_{II-4}) subunits, which act as hydrophobic membrane anchors, is a general feature of mitochondrial complex II (*11*, *12*). In addition, the anchor subunits in complex II are essential for the interaction between the complex and quinone species (*11*).

In these studies, we attempted to elucidate the molecular basis of a mitochondrial disease, such as Leigh syndrome, caused by a deficiency in the SQR activity of complex II. Therefore, we cloned the cDNAs for all four subunits of human liver complex II and identified their unique features (*13–15*). All of the human complex II genes are encoded on nuclear DNA, and we mapped the genes for cybL and cybS, *SDHC* and *SDHD*, to chromosomes 1q21 and 11q23, respectively (*15*). The genes for Fp (*SDHA*) and Ip (*SDHB*) have been mapped to chromo-

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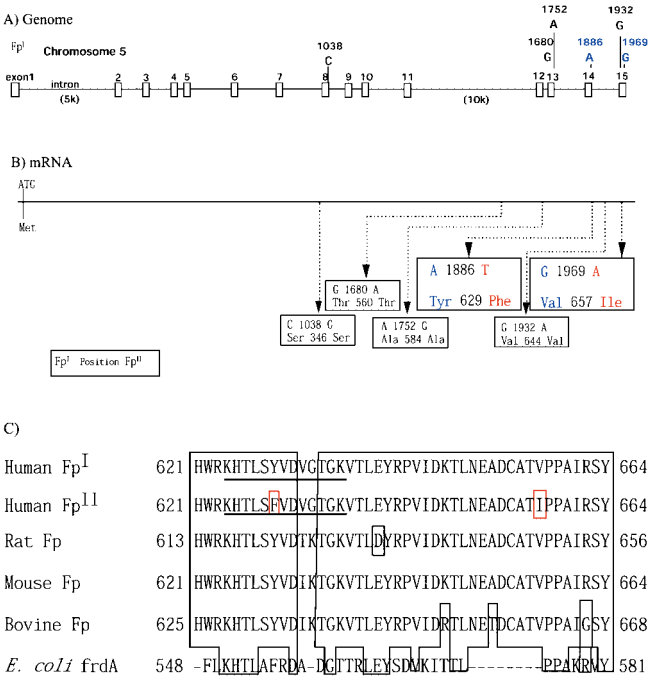

Fig. 1. **Differences in nucleotides and amino acids in chromosome 5p15 and cDNA sequences for the Fp subunit of human complex II.** (A) The Type I Fp sequence is located in chromosome 5p15 complementary, while the Type II Fp sequence was not found in the chromosomal database. (B) Type II Fp differs from Type I in 6 nucleotides and 2 amino acids. (C) Comparison of the C-terminal amino acid sequences of Fp subunits from various species. Human Fp^{I} and Fp^{II} [this study]; Rat Fp [NP_569112]; mouse Fp [XP_127445]; Bovine Fp [NP_776603]; *E. coli* frd [NP_418578]. The similarity in the sequences of the various species was maximized with the computer software GENETYX. The peptides with the amino acid sequence of Type I (KHTLS**Y**VDVGTGK; sonar:3.4E-5) and that of Type II (KHTLS**F**VDVGTGK; XCORR:2,77,2+) found in Supplementary Table 3 in Ref. *18* are underlined.

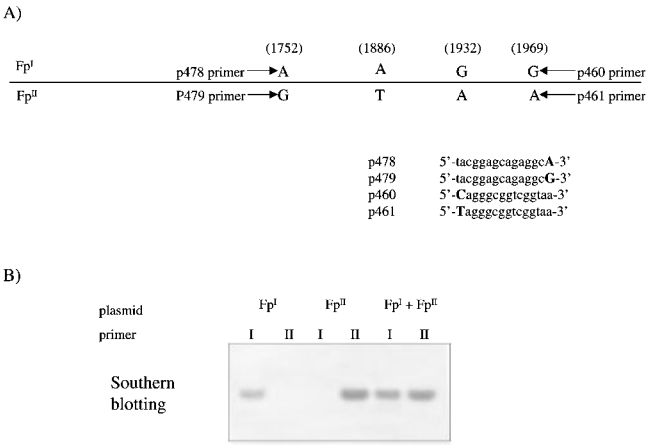

Fig. 2. **PCR of cDNAs using Fp type-specific primers.** (A) Localization of primers and the designs of the specific primers. (B) PCR for plasmids with Type I Fp and Type II Fp inserts. The Fp bands amplified from the plasmids containing Type I and Type II Fp inserts correspond to 246 bp. An Fp band was not detected for the Type II Fp plasmid using the Type I Fp primers (p478/p460) nor was a band detected for the Type I Fp plasmid using the Type II Fp primers (p479/p461).

somes 5p15 (*3*) and 1p35–36.1 (*16*) by other groups. Comparison of the cDNA sequences of liver Fp (named Type I Fp), a subunit that we previously described (*14*), and heart Fp (named Type II Fp), which was cloned by Morris *et al.* (*17*), revealed differences in 6 nucleotides and 2 amino acids (Fig. 1). Our cDNA sequence for liver complex II was identical to the sequence of a mRNA predicted from the genomic sequence of *SDHA* on chromosome 5p15, while no DNA sequence corresponding to the heart Fp was found. The *SDHA* on chromosome 5p15 comprises 15 exons extending over 38 kb encoding a 664 amino acids. As shown in Fig. 1, the two different amino acids between Type I Fp and Type II Fp are located in exons 14 and 15, which encode the C-terminus of Fp. These results suggest the possibility of multiple Fp isoforms. In the present study, we identified two distinct cDNAs for human Fp from a single individual as described below.

To validate the presence of two distinct human Fp cDNAs, we examined cDNA libraries from human muscle (STRATAGENE #936215) and liver (STRATAGENE #937241) mRNAs that were isolated from different individuals. These libraries were screened with a monoclonal antibody against *Ascaris suum* Fp, which recognizes the conserved amino acids Glu603-Tyr606 of mitochondrial Fp. Two different cDNAs were obtained from both cDNA libraries. Sequence analysis of the full-length cDNAs revealed that one was identical to the Fp cloned from liver (Type I Fp) and the other was identical to the Fp from heart (Type II Fp). Of the positive clones from muscle, 10% were Type I Fp, while Type I Fp accounted for 67% of the positive clones from liver. These results confirm the presence of two distinct human Fps.

We further analyzed the tissue expression of the two Fps by RT-PCR. We designed several primer sets for the specific amplification of the mRNA region that encodes the two different amino acids. Two of the primer sets amplified Type I and II Fps specifically from template plasmids containing their respective cDNAs (Fig. 2B). Nearly the same level of amplification was observed for Type I and II Fps when the template DNA included cDNAs for both Fps. This result shows that the two primer sets were able to concurrently amplify Type I and II cDNAs in a single reaction. Therefore, we used these primer sets to examine the expression of the two Fps in various organs. The mRNAs of skeletal muscle, liver and brain were isolated from different individuals, while those of heart and kidney were pooled materials. As shown in Fig. 3, both Type I and II Fps were expressed in all the organs analyzed. Type I Fp was expressed at a higher level than Type II in all cases. Identical results were obtained for muscle biopsy samples from 5 healthy Japanese volunteers (data not shown). In contrast to these findings, cultured myoblasts and fibroblasts expressed only Type I Fp (Fig. 3).

Because the Type II Fp sequence was not found in the Genome Projects database, we analyzed human genomic DNA using the primer set that amplifies the DNA fragment containing exons 14 and 15 (Fig. 4). With this primer set, we amplified a 2 kbp fragment comprised of *SDHA* on chromosome 5p15 and a pseudogene of Fp on chromosome 3q29 that contains a 1-bp deletion creating a frame shift (*4*). In addition to this 2 kbp band, we consist-

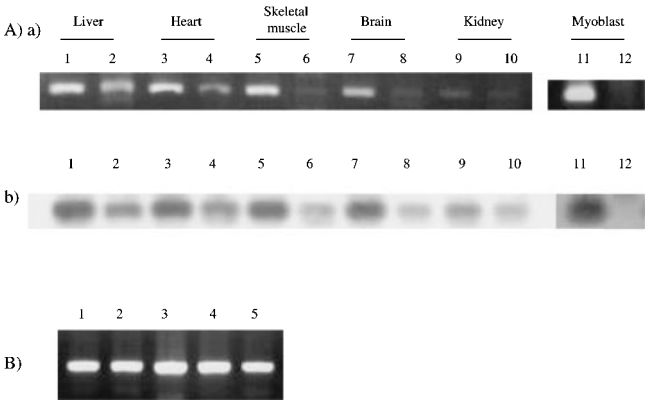

Fig. 3. **RT-PCR analysis using Fp type-specific primers.** (A) PCR analysis of human tissue cDNAs using type-specific primers. The cDNAs were obtained from NIPPONGENE. Skeletal muscle #312-04231, Lot No. 01000G mRNA pool of one tissue, male, 23 years old. Liver #318-04071, Lot No. 02009L mRNA pool of one tissue, female, Caucasian, 15 years old. Brain #317-04041, Lot No. 03000E mRNA pool of one tissue, male, Caucasian, 50 years old. Heart #314-04051, Lot No. 02000A mRNA pool of seven tissues, male/female, Caucasian, 20–78 years old. Kidney #313-03661, Lot No. 07000E mRNA pool of eight tissues, male/female, Caucasian, 24–55 years old. cDNAs were also isolated from human cultured myoblasts and fibroblasts. The PCR reaction was carried out for 35 cycles with denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min. (a) Shown is an ethidium bromide-stained 1.5% agarose gel displaying cDNA amplified with human Type I and II Fp primers from cDNA from human liver, heart, skeletal muscle, brain and kidney. Lanes 1, 3, 5, 7, 9, and 11: amplified with Type I–specific primers. Lanes 2, 4, 6, 8, 10, and 12: amplified with Type II-specific primers. (b) Southern blotting from (a). Human Fp cDNA was used as the hybridization probe. (B) As a positive control, amplification of a fragment of the human glyceraldehyde-3-phosphate dehydrogenase mRNA was performed, using a sense-primer (5′-CCTTCATTGACCTCAACTA-3′) and an antisense-primer (5′-GCCAGTGAGCTTCCCGTTCA-3′) selected on the basis of the GenBank cDNA sequence (accession No. NM002046). The PCR reaction was carried out for 35 cycles with denaturation at 95°C for 30 sec, annealing at 59°C for 30 s, and extension at 72°C for 1 min. Shown is an ethidium bromide-stained 1.5% agarose gel displaying cDNA amplified with glyceraldehyde-3-phosphate dehydrogenase-specific primers. Lane 1: cDNA of human liver; lane 2: heart; lane 3: skeletal muscle; lane 4: brain; lane 5: kidney.

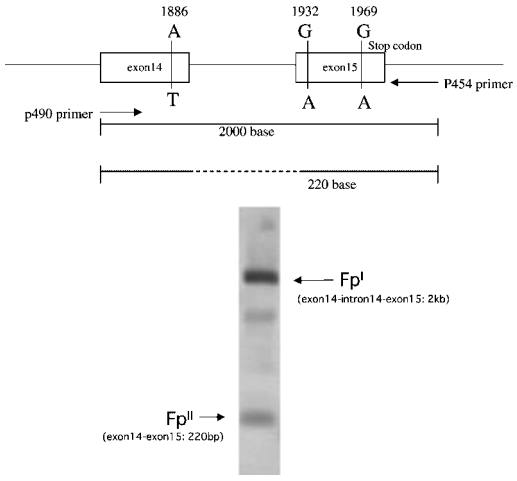

Fig. 4. **Detection of exon-intron Fp and intronless Fp by Genomic PCR analysis.** The PCR reaction was carried out for 32 cycles with denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2.5 min using the p490 primer (5′-ATGAGTACGATTACTCCAAGC-3′) and the p454 primer (5′-TCACCACATCTTGTCTCATCA-3′). Southern blotting was performed following 1% agarose gel electrophoresis. Human Fp cDNA was used as the hybridization probe.

ently observed a 220 bp DNA fragment in Southern blots. We isolated and sequenced this DNA because it corresponded to the predicted size of the fragment from Fp cDNA. DNA sequencing revealed that the 220 bp fragment contained both Type I and Type II Fps, suggesting the presence of intronless copies on the chromosome.

To characterize the intronless copies further, amplification of a much longer fragment was attempted using primers based on the upstream exons and the region downstream of the termination codon in *SDHA*. All of the intronless clones of Type I Fp lacked stop codons, suggesting that the intronless copies of Type I are a pseudogene (data not shown). In contrast, the nucleotide sequence of the Type II intronless clones containing exons 5 to 15 was identical to the Type II Fp. We found a poly A sequence in the 3′terminus of the intronless gene, and the 259 bp 3′UTR sequence was identical to those in the Type I and II Fp cDNAs. These results indicate that the Type II Fp gene has no intron in the coding region and might have been produced by a retrotransposition of the original Fp gene.

Our results clearly demonstrate the presence of two distinct Fps, Types I and II, in human complex II. Both types of Fp were expressed in liver, heart, skeletal muscle, brain and kidney, although expression of Type I was higher than that of Type II in all organs examined. During preparation of this manuscript, proteomic analysis of human heart mitochondria was reported (*18*). The peptides corresponding to the amino acid sequences of Type I (KHTLS**Y**VDVGTGK; sonar:3.4E-5) and II (KHTLS**F**VDVGTGK; XCORR:2,77,2+) were identified by LC-MS/MS analysis of the mitochondrial proteins, confirming our finding of two types of Fp (see Supplementary Table 3 in Ref. *18*). The Type II Fp gene has not been found in the public human genome database (NCBI on 2003. June 13), while that for Type I is located on chromosome 5p15. In addition to *SDHA* on chromosome 5, there are several pseudogenes of Fp, including two tandem pseudogenes with a 1-bp deletion located on chromosome 3q29. Fragments containing the DNA sequences from exons 2–6 and exons 10–15 are found on chromosome 3 (within 200 kb of two pseudogenes) and 1.6 Mb adjacent to *SDHA* on chromosome 5. It is possible that the intronless Type II gene is located in the telomeric region of chromosomal DNA because it was not found in the public database and because this region is sometimes difficult to sequence (*19*). The presence of intronless copies is not surprising because several housekeeping genes have functional second genes generated by retrotransposition (*20*).

Three cases have been reported of a nuclear mutation in the gene encoding Fp (*3*, *4*, *21*). The first report described two siblings with Leigh syndrome that was associated with a deficiency of SQR activity due to the homozygous substitution Arg544Trp. Because nucleotide 1680 of the patient's Fp gene is A, this mutation appears to be in Type II Fp (see Fig. 1). Another case of Leigh syn-

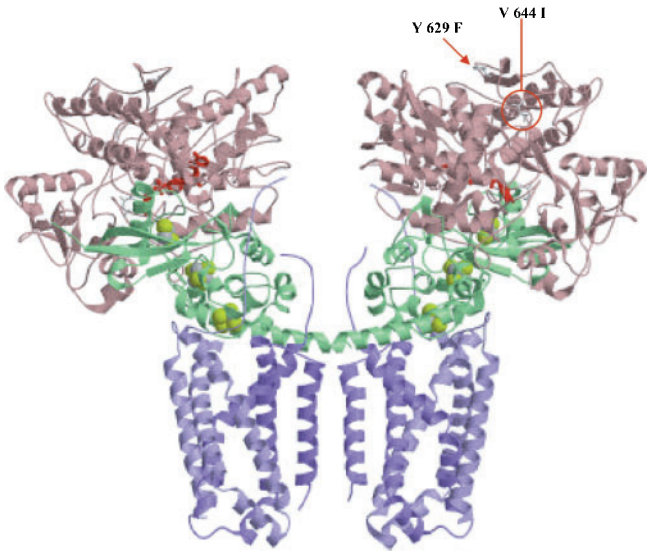

Fig. 5. **The crystal structure of fumarate reductase (complex II) in *Escherichia coli* (*28*).** Amino acid residues different between Type I and II Fp are superimposed, specifically F 554 (Human: Y 629 F), which resides in the β sheet of K550 to F554, and L 573 (Human: V 644 I).

drome was associated with the heterozygous substitution Ala524Val, inherited from the father, and Met1Leu from the mother (*4*). The Fp mRNA of this patient was very unstable. In a third case, two sisters exhibited a partial decrease in SQR activity, which was associated with late-onset optic atrophy, ataxia and myopathy (*21*). They carried a heterozygous Arg408Cys substitution. The latter two mutations could not be detected in either of the two types of Fp because sufficient DNA sequence information for these patients was not available.

The present study is the first report demonstrating the existence of two distinct complex IIs in mammalian mitochondria. Similarly, some nematodes possess more than two complex IIs. Complex II from the adult *A. suum* mitochondria exhibits high quinol-fumarate reductase (QFR) activity and functions as a terminal oxidase in the NADH-fumarate oxidoreductase system of anaerobic metabolism. In contrast, complex II from free-living third stage larvae (L3) has lower QFR activity and functions as a succinate dehydrogenase (SDH) during aerobic respiration. Thus, complex II plays a key role in the two different energy metabolism systems of the parasitic nematode, *A. suum*. A recent study showed that both the Fp and CybS subunits are different in the larval and adult complex IIs, while the Ip and CybL subunits are shared by the two forms of *A. suum* complex II (*22*, *23*). The well-known free-living nematode, *Caenorhabditis elegans*, also has two Fp genes, although their specific functions are not clear. The genes for Fp-I and Fp-X of *C. elegans* are located on chromosomes I and X, respectively, and expression of both gene products was demonstrated by *in situ* hybridization and Western blot (Amino *et al.*, unpublished observation).

Two of the ten nuclear-coded subunits of human complex IV (cytochrome *c* oxidase), VIa and VIIa, occur as tissue-specific isoforms (*24*). The heart-type (VIaH, VIIaH) is expressed primarily in adult heart and skeletal muscle, whereas the liver-type (VIaL, VIIaL) is expressed in all non-heart/skeletal muscle tissues. Fetal heart and skeletal muscle mainly express the liver-type isoforms, switching to the heart-type after birth (*25*). Although it is difficult to determine which type of Fp is essential in the human mitochondria and is important for the tissue-specific clinical pathologies of patients with Fp deficiencies, Type I Fp may be the major Fp subunit because of its higher mRNA expression. Consistent with this possibility, the two amino acids specific to Type I Fp are more highly conserved than those in Type II (Fig. 1C). Because there is only a two amino acid difference between human Type I and II Fps, it is likely that the basic properties of the two enzymes might be similar. However, it is clear that active Type II Fp is essential for energy metabolism in human mitochondria because the mutation of Fp in the first case of Leigh syndrome was in the Type II protein (*3*). This difference may not effect the interaction between Fp and Ip or assembly of the complex because the two amino acids are in the C-terminus of Fp, a region that is not in direct contact with the other subunits (Fig. 5). Instead, the difference between Type I and II Fps might affect the catalytic process, including substrate binding, because the C-terminal domain folds around the core structure and active site and plays an important role in the opening and closing of the active site (*26*). We therefore speculate that, like *A. suum* adult complex II, one of the mammalian isoforms has fumarate reductase activity and plays a role in anaerobic metabolism during hypoxia. If this were the case, succinate would be expected to accumulate as an end product of ATP generation *via* anaerobic α-ketoglutarate/aspartate metabolism. In support of this possibility, succinate is known to accumulate in kidney proximal tubule cells during hypoxia (*27*). Our current studies are examining this possibility, and we are performing further biochemical studies to understand the physiological functions of the two forms of complex II in human mitochondria.

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