Very-long-chain acyl-CoA dehydrogenase subunit assembles to the dimer form on mitochondrial inner membrane

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Abstract This paper describes the process of dimer assembly of mitochondrial very-long-chain acyl-CoA dehydrogenase (VLCAD) subunit. Mature VLCAD is a homodimer of a 70-kDa protein associated with the mitochondrial membrane. Newly synthesized VLCAD was present as a monomer and the major fraction was associated with the mitochondrial inner membrane. The association of VLCAD subunit with the mitochondrial membrane was observed early during dimer formation. In contrast, a VLCAD monomeric mutant S583W, a novel mutation identified from a patient with VLCAD deficiency, did not associate with the mitochondrial membrane after import and the major fraction remained in the mitochondrial matrix. These results suggest that association of VLCAD protein with mitochondrial inner membrane is necessary for dimer assembly and formation of mature VLCAD.

Key words: Very-long-chain acyl-coenzyme A dehydrogenase; Mitochondrial import; Dimer assembly; Mitochondrial inner membrane

1. Introduction

Very-long-chain acyl-CoA dehydrogenase (VLCAD) is one of four enzymes which catalyze the initial step of mitochondrial fatty acid β-oxidation spiral. These enzymes have overlapping substrate-chain-length specificities. Three of the acyl-CoA dehydrogenases are mitochondrial matrix proteins and homotetramers of approximately 40-kDa polypeptides, whereas VLCAD is an inner membrane-associated protein and a homodimer of a 70-kDa polypeptide [1]. The amino-terminal region of VLCAD shows significant sequence homology to other acyl-CoA dehydrogenases. However, the carboxyl-terminal has a long tail with approximately 180 amino acid residues, which is not shared by other acyl-CoA dehydrogenases [2,3].

We have studied the molecular basis of VLCAD deficiency. This disease is a relatively recently identified inborn error of the mitochondrial fatty acid oxidation system resulting in a spectrum of findings noted in fatty acid oxidation disorders. Clinical findings noted include hypertrophic cardiomyopathy, skeletal myopathy, hypoglycemic hypoketonuria and sudden infant death syndrome [4–8]. We previously identified 6 mutations in VLCAD cDNAs from 6 patients with this disease [3,9].

The mutations identified were shown to result in decreased VLCAD activity in expression studies [9]. Post-translational modification of VLCAD polypeptide to form the normal VLCAD homodimer was impaired. Translational products of the mutations located at the amino-terminal region (E130del, K299del and K382Q) showed some degree of the dimer formation, whereas R613W, only a mutation in the carboxyl-terminal tail, was monomeric. We report the identification of a novel monomeric mutation in the carboxyl-terminal sequence of VLCAD. In an attempt to further characterize the process of VLCAD dimerization and the function of the carboxyl-terminal sequence, we studied the process of dimer assembly of native and a carboxyl-terminal mutant VLCAD polypeptide.

2. Materials and methods

2.1. Patient

A female patient presented at 3 months of age with an acute episode of hypoketotic hypoglycemia and hypotonia. In addition to the generalized hypotonia, marked hepatomegaly was noted. Evaluation at that time revealed elevations in long chain acylcarnitine levels and dicarboxylic aciduria. In skin fibroblasts from this patient, the palmitoyl-CoA dehydrogenase activity was 0.37 mU/mg, which was significantly decreased compared to that in control (2.10 ± 0.31 mU/mg), and cross-reactive protein with anti-VLCAD antibody was hardly detected (data not shown).

2.2. Cell lines and cell culture

Chinese hamster ovary (CHO) cells transfected with pCXN2 vector including VLCAD cDNA were previously described [9]. Skin fibroblasts from the patient and CHO cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 0.1 mM non-essential amino acids (Gibco/BRL Oriental), 1× antibiotic-antimycotic solution (Gibco/BRL Oriental), and 4.5 mg/ml glucose.

2.3. Cell labeling and gel-filtration analysis

The transfected cells were grown in 100-cm^2 dishes. Before labeling, the cells were incubated for 60 min in methionine-free modified Eagle's medium containing 5% (v/v) dialyzed fetal bovine serum. The medium was replaced with the same medium containing 0.1 mCi of [^{35}S]methionine (Amersham Co., Amersham, UK) and the cells were incubated for 10 min. In the case of chase, the medium was replaced with a standard medium and the cells were further incubated for 20 min. The labeled cells were harvested and sonicated with 20 mM potassium phosphate/0.15 M NaCl/0.2% Tween 20/1 mM PMSF. The suspension was centrifuged at $100\,000 \times g$ for 30 min, and the supernatant was applied to a G3000SW column (7.5 mm I.D.×60 cm; Tosoh, Tokyo, Japan) equilibrated with 20 mM potassium phosphate (pH 7.5)/0.15 M NaCl/0.2% Tween 20. The elution was performed with the same buffer at a flow rate of 1 ml/min, and each of 1 ml eluates was collected. Fractions were subjected to immunoprecipitation, SDS-PAGE and fluorography.

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Abbreviations: VLCAD, very-long-chain acyl-CoA dehydrogenase; hsp60, heat shock protein 60

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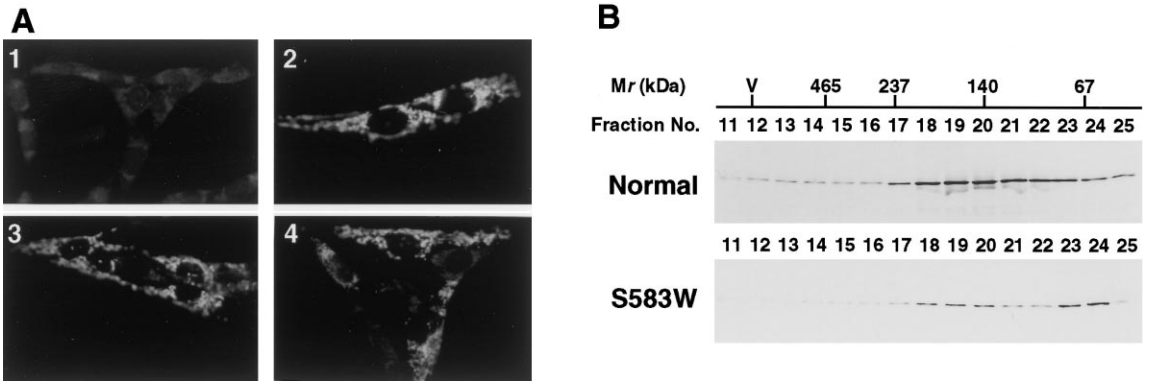

Fig. 1. Expression of VLCAD and S583W in CHO cells. A: immunofluorescence microscopy of VLCAD- and S583W-transfected CHO cells using anti-VLCAD antibody. Panel 1: untransfected cells; panel 2: VLCAD-transfected cells; panel 3: S583W-transfected cells; panel 4: CHO cells stained with Rhodamine 123. B: gel-filtration analysis. Protein standards used: *E. coli* β-galactosidase (465 kDa), rabbit muscle pyruvate kinase (237 kDa), rabbit muscle lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa).

2.4. Other methods

Methods for RNA isolation, PCR amplification, sequencing, vector construction and transfection of CHO cells were described previously [9].

3. Results and discussion

3.1. Expression of a novel mutant VLCAD

A C-1748-to-G transversion, which changes Ser-583 to Trp (S583W), was identified in PCR-amplified cDNA from the patient. Analysis of the family members for this mutation revealed that the patient was a compound heterozygote with the other allele having another mutation which could not be identified in the present study because of significantly decreased level of its mRNA.

We performed cDNA-expression experiments of the novel mutation using CHO cells. The palmitoyl-CoA dehydrogenase activity in untransfected, VLCAD- and S583W-transfected cells were 2.37, 12.3 and 2.29 mU/mg, respectively. Fig. 1A shows the immunofluorescence microscopy of untransfected (panel 1), VLCAD- (panel 2) and S583W-(panel 3) transfected cells. In the untransfected CHO cells, the signal was hardly detected. On the other hand, an intense dotted fluorescence pattern was observed in both VLCAD- and S583W-transfected cells, indicating the expression of these proteins in the cells. Similar staining of the cells was observed using Rhodamine 123, which stains mitochondria [10] (panel 4). Thus, both the expressed VLCAD and S583W proteins were located in mitochondria of the host cells. Mitochondrial import of these proteins was also confirmed by the cell fractionation analysis with proteinase treatment of separated mitochondria (data not shown).

Fig. 1B shows the gel-filtration analysis of VLCAD and S583W proteins expressed in CHO cells. Expressed VLCAD was mainly eluted in fractions 19 and 20, of which the molecular mass was estimated to be 150 kDa, corresponding to that of the purified enzyme. On the other hand, S583W protein was mainly detected in fractions 23 and 24, of which the molecular mass was estimated to be 70 kDa, corresponding with that of the monomeric subunit of VLCAD. The results of gel-filtration analysis indicate that majority of soluble S583W protein is present as a monomer in the host cells, suggesting that the process of dimer assembly after normal import into mitochondria is impaired. These observations for S583W were similar to those for the previously identified mutation, R613W [9].

3.2. Dimer assembly of VLCAD

To study the process of dimer assembly of VLCAD, the

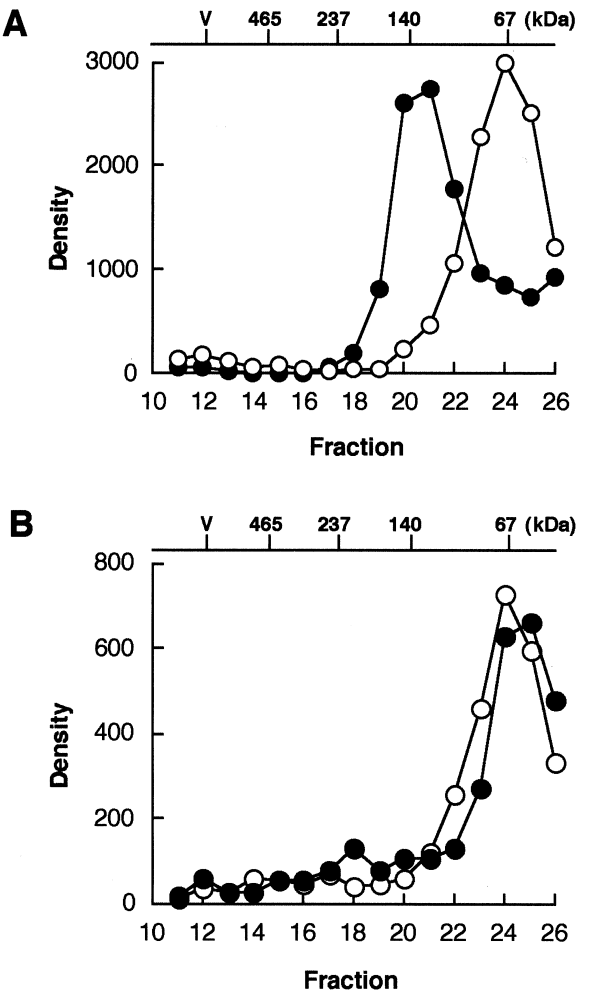

Fig. 2. Gel-filtration analyses of 10-min labeled (○) and 20-min chased cells (●). The result of fluorography was quantified and plotted. A: VLCAD; B: S583W. V indicates the void fraction.

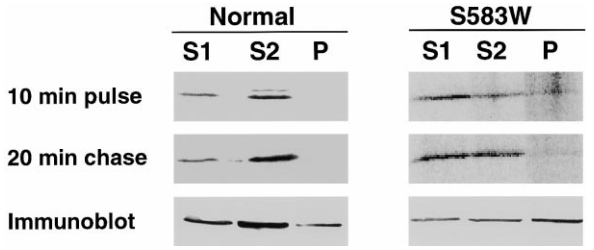

Fig. 3. Solubility of newly imported VLCAD. The cells were sonicated with 20 mM potassium phosphate (pH 7.5)/1 mM EDTA/1 mM PMSF, and centrifuged at 100 000×*g* for 30 min. The supernatant (S1) was moved to another tube and 0.15 M NaCl and 1% Triton X-100 were added. The pellet was resuspended with 20 mM potassium phosphate (pH 7.5)/0.15 M NaCl/1% Triton X-100 and sonicated, then centrifuged at 100 000×*g* for 30 min. The supernatant (S2) was transferred to another tube, and the pellet (P) was resuspended in the same buffer. The labeled VLCAD in each fraction was collected by immunoprecipitation, and subjected to SDS-PAGE and fluorography. Immunoblotting of the fractions was also done.

transfected cells were briefly labeled with [^{35}S]methionine and labeled VLCAD was analyzed by gel-filtration chromatography. In cells labeled for 10 min, one peak of ^{35}S-VLCAD was found in fractions 23–25 (Fig. 2A). Newly synthesized (10 min-labeled) VLCAD was confirmed to be rapidly imported into mitochondria, as it was mainly detected at the mature size on SDS-PAGE/fluorography. In addition, newly synthesized VLCAD was not degraded by the proteinase treatment of separated mitochondria (data not shown). After chase for 20 min, the major peak of ^{35}S-VLCAD was observed in fractions 19–21, corresponding to the dimer with comparable density as that in the monomer fraction in the cells that were labeled for 10 min. These results indicate that newly imported VLCAD is present as a monomer followed by dimer assembly, which occurs time dependently.

It is known that several newly imported mitochondrial proteins form a complex with heat shock protein 60 (hsp60), which promotes folding and assembly, and the release from the complex with hsp60 requires ATP [11–13]. Saijo et al. [14] demonstrated by in vitro translation/import experiments that the folding and tetramer assembly of medium-chain acyl-CoA dehydrogenase, a mitochondrial matrix protein, proceeds ATP-dependently after formation of a high molecular weight complex with hsp60 in mitochondria. Since the presence of hsp60 in CHO cell mitochondria has been reported [15,16], we expected that newly imported VLCAD would be found in high molecular weight fractions if the dimer assembly of VLCAD also required hsp60. However, no signal was detected in high molecular weight fractions (fractions 11–20), which would correspond to the predicted hsp60-acyl-CoA dehydrogenase complex (Fig. 2A). Additionally, cDNA-expressed VLCAD could form a homodimer, even if hsp60 was absent in CHO cells. This result suggests that hsp60 does not contribute to the dimer assembly of VLCAD. Since the organization and structure of other mature acyl-CoA dehydrogenases and VLCAD are different, the mechanism of complex assembly is different.

In a strain of yeast defective in mitochondrial hsp60 function, mitochondrial matrix-tergeted proteins were misfolded and/or defective for import and sorting, whereas intramembrane space proteins such as cytochrome b_2 and c_1 and inner membrane proteins such as cytochrome *c* oxidase subunit Va and ADP/ATP carrier were normally imported and assembled into the functional form [17,18]. Thus, hsp60 is likely to be required for the matrix proteins in their folding and assembly, but not for the intramembrane space and inner membrane proteins.

3.3. Membrane association of newly imported VLCAD

Intact VLCAD is a mitochondrial inner membrane associated protein, although other acyl-CoA dehydrogenases are matrix proteins. We examined the membrane association of newly imported VLCAD subunit. The 10-min labeled or 20-min chased cells were fractionated as soluble fractions without (S1) and with 1% Triton X-100 (S2) and insoluble fraction (P). In VLCAD transfected cells, most of 10-min labeled VLCAD was detected in S2 fraction (Fig. 3), suggesting the membrane association of newly imported VLCAD. The solubility of VLCAD did not change after chase for 20 min and at a steady state determined by immunoblotting. In contrast, ^{35}S-S583W in both 10-min labeled and 20-min chased cells, which existed as a monomer (Fig. 2B), was mainly found in S1 fraction (Fig. 3). The same results were observed in the other monomeric mutant R613W (data not shown). Thus, membrane association of VLCAD subunit appears to be required for the process of dimer assembly. Recently, some of the proteins which have chaperone-like activity in mitochondrial inner membrane have been identified [19]. Dimer processing and assembly of VLCAD may be a function of or facilitated by these inner membrane proteins.

A significant amount of S583W protein was insoluble at a steady state, although the newly imported S583W was soluble (Fig. 3). The half-life of S583W protein was within 6 h, although that of normal VLCAD was more than 24 h. Also, we previously identified four labile mutant VLCAD [9], and confirmed that significant amounts of these translational products were insoluble (unpublished observation). Insolubility of these mutant proteins may relate to their rapid degradation, perhaps including the complex formation with the degradation factors.

These results indicated that VLCAD protein associates with the mitochondrial inner membrane and that dimerization to form mature VLCAD occurs there. The S583W protein did not associate with the inner membrane, raising the possibility that the change in amino acid sequence interferes with normal localization of the protein within the mitochondria. Whether the localization of VLCAD is determined by the 180 amino acid carboxyl-terminal sequence has not been determined. These results would suggest a possible role for this sequence and distinguish VLCAD from the other fatty acid acyl-CoA dehydrogenases. The specific steps and proteins involved in localization and dimerization remain to be identified.

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