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Is Antiquitin a Mitochondrial Enzyme?

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ABSTRACT

Antiquitin is an aldehyde dehydrogenase involved in the catabolism of lysine. Mutations of antiquitin have been linked with the disease pyridoxine-dependent seizures. While it is well established that lysine metabolism takes place in the mitochondrial matrix, evidence for the mitochondrial localization of antiquitin has been lacking. In the present study, the subcellular localization of antiquitin was investigated using human embryonic kidney HEK293 cells. Three different approaches were used. First, confocal microscopic analysis was carried out on cells transiently transfected with fusion constructs containing enhanced green fluorescent protein with different lengths of antiquitin based on the different potential start codons of translation. Second, immunofluorescence staining was used to detect the localization of antiquitin directly in the cells. Third, subcellular fractionation was carried out and the individual fraction was analyzed for the presence of antiquitin by Western blot and flow cytometric analyses. All the results showed that antiquitin was present not only in the cytosol but also in the mitochondria. J. Cell. Biochem. 109: 74–81, 2010.

KEY WORDS: ALDEHYDE DEHYDROGENASE; ANTIQUITIN; PYRIDOXINE-DEPENDENT SEIZURES; SUBCELLULAR LOCALIZATION; MITOCHONDRIA

Antiquitin is a member of the aldehyde dehydrogenase (ALDH) superfamily [Vasiliou and Nebert, 2005; Fong et al., 2006]. It was first discovered about two decades ago when its expression was found to be significantly elevated in dehydrated green garden pea [Guerrero et al., 1990]. Since the amino acid sequence is highly homologous for human and plant antiquitins, this protein was given the name "antiquitin" to reflect its antique nature [Lee et al., 1994]. While plant antiquitin is believed to be involved in the response against oxidative and/or osmotic stress [Stroeher et al., 1995; Oztur et al., 2002; Kirch et al., 2005; Moulin et al., 2006], its human counterpart did not show any inducibility against various types of challenges [Lee et al., 1994; Wong et al., 2006]. The physiological function of human antiquitin was only unraveled in 2006 when it was discovered that mutations of antiquitin are responsible for pyridoxine-dependent seizures [Mills et al., 2006; Plecko et al., 2007; Striano et al., 2009]. Antiquitin acts as the enzyme catalyzing the oxidation of α-aminoadipic semialdehyde (α-AASA) in the lysine catabolic pathway [Mills et al., 2006]. Mutations of antiquitin lead to a deficiency of enzyme activity that not only accumulates α-AASA, but also its equilibrium product Δ^1-piperideine-6-carboxylate. The latter will undergo Knoevenagel condensation with pyridoxal-5-phosphate, leading to a deficiency of Vitamin B6 for the neurotransmission process.

Although the physiological role of human antiquitin has been clarified, relatively little is known about the biochemical properties of the human protein, except a detail structure obtained by X-ray crystallography (PDB: 2J6L). On the other hand, more is known about the enzyme of other vertebrates. Seabream antiquitin has been purified from the fish liver [Tang et al., 2002], expressed in a bacterial system [Tang et al., 2005], and kinetically characterized. Structural analyses have also been performed and the amino acid residues involved in the substrate binding pocket were identified by site-directed mutagenesis [Tang et al., 2008]. The seabream enzyme was believed to be a cytosolic enzyme based on the results of fluorescent microscopic analysis of cells transiently transfected with antiquitin fused with enhanced green fluorescent protein (EGFP) [Tang et al., 2005]. For the human enzyme, a preliminary study by subcellular fractionation also indicated its cytosolic nature [Chang et al., 1990].

The cytosolic localization of antiquitin is not consistent with its presumed physiological role in lysine metabolism which is known to occur in the mitochondrial matrix [Blemings et al., 1994]. Thus, a re-evaluation of the subcellular localization of antiquitin is highly warranted. This is particularly important in view of the fact that human antiquitin possesses more than one potential start codon. Utilization of different start codons may result in different

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TABLE I. Nucleotide Sequence of the Forward and Reverse Primers Used for Preparing the Various Constructs of Human Antiquitin

Constructs	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Ampicon size (bp)
FL	TgAgCCCTCgAgATgTCCACTCTCCTCATC	ggCTCAgAATTCgCTgAAACTTgATT	1,533
SP	TgAgCCCTCgAgATgTggCgCCTTCCTCgC	ggCTCAgAATTCggAAggCggCAggCCT	84
SPFL	TgAgCCCTCgAgATgTggCgCCTTCCTCgC	ggCTCAgAATTCgCTgAAACTTgATT	1,617

subcellular localization of protein [Claudiani et al., 2005; Rentero and Puigdomenech, 2006]. In the present investigation, attempts were made to clarify the subcellular localization of human antiquitin by three different approaches, viz. confocal microscopy of cells transfected with different antiquitin-EGFP constructs, immunofluorescence, and finally, Western blot and flow cytometric analyses of antiquitin in isolated subcellular fractions.

MATERIALS AND METHODS

CELL CULTURE

HEK293 cells were cultured in Dulbecco's Modified Eagles's medium (DMEM) supplemented with 10% heat-inactivated horse serum (v/v), 1% non-essential amino acid (v/v) and 1% penicillin-streptomycin (v/v). Cells were cultured at 37°C in a humidified incubator containing 5% CO_2. All the cell culture materials used were purchased from Invitrogen.

CONFOCAL MICROSCOPY OF CELLS TRANSIENTLY TRANSFECTED WITH DIFFERENT ANTIQUITIN-EGFP CONSTRUCTS

All the primers used were purchased from Invitrogen, and designed to have *Xho*I and *EcoR*I cutting sites for cloning into the pEGFP.N1/C3 vectors. A cDNA plasmid of human antiquitin with sequence as published in the NCBI GenBank in 2003 (NM_001182.1, FL, 1533 bp) was purchased from Clontech. The signal peptide (SP) sequence obtained from the NCBI GenBank (NM_001182.3, 193rd–276th nucleotides, Fig. 1) was purchased from Invitrogen. It was amplified by PCR using primers in Table I. All the constructs were verified by sequencing. HEK293 cells (4×10^5) were seeded on a 0.4% gelatin coated confocal dish (MatTek). Transfection of cells was performed using Lipofectamine (Invitrogen) according to the manufacturer's specifications. After 48 h, the transiently transfected cells were stained with 500 nM of MitoTracker Red CMX-Ros (Invitrogen) for 45 min and the nuclei were stained with 1 mM Hoechst33342 (Invitrogen) for 15 min in the culture medium at 37°C in a humidified incubator containing 5% CO_2. Cells were washed twice with phosphate-buffered saline (PBS) before visualization under a Leica SP5 confocal microscope.

IMMUNOFLUORESCENCE STAINING OF ANTIQUITIN IN CELLS

HEK293 cells were cultured on a confocal dish and labeled with MitoTracker as mentioned. They were then fixed and permeabilized with ice-cold methanol for 30 min at 4°C. The cells were pre-incubated with 0.2% bovine serum albumin in a high potassium buffer (80 mM HEPES, 5 mM EDTA, 2 mM $MgCl_2$, 0.1% Triton X-100, pH adjusted to 7 by KOH) for 15 min at room temperature to block any non-specific binding. They were then incubated with the primary antibody (1:200) against human antiquitin (rabbit antibody from Epitomics) or cytochrome *c* oxidase subunit IV (CoxIV) (mouse antibody from Abcam) in the same buffer for 16 h at 4°C. After rinsing with buffer, the cells were incubated with the corresponding secondary goat anti-rabbit or anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC, Invitrogen) or Alexa Fluor 488 (Invitrogen) (1:200) for 4 h at room temperature. The cells were washed with the buffer twice and resuspended in PBS before visualized under a Leica SP5 confocal microscope.

SUBCELLULAR FRACTIONATION AND WESTERN BLOT ANALYSIS

HEK293 cells were collected, washed once with PBS, resuspended in RIPA buffer (1% NP-40, 5% sodium deoxycholate, 0.1% SDS, in PBS), and incubated on ice for 10 min to obtain the whole cell lysate. Lysates were then centrifuged at 20,000*g* for 7 min. The supernatant collected was taken as the total protein.

Fractionation to separate particulate and cytosolic proteins was carried out using the Cytosol/Particulate Rapid Separation Kit (BioVision) according to the manufacturer's protocol. Briefly, cells were collected by centrifugation at 600*g* for 5 min at 4°C. The cells were resuspended in cell suspension buffer and were lysed with

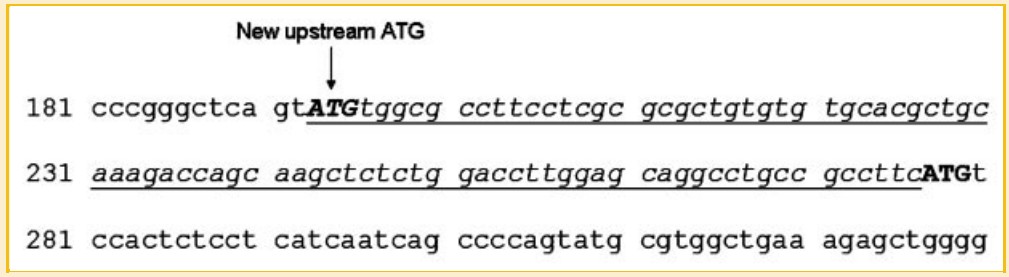

Fig. 1. Location of the SP at the N-terminal of human antiquitin. Nucleotide sequence (GenBank Accession no. NM_001182.3) showed the additional upstream potential start codon in human antiquitin.

cytosol releasing buffer for 30 s. The tube was spun at 20,000*g* for 1 min. The cytosol and particulate fractions could be physically separated by the middle oil layer. The cytosol fraction (top layer) was collected while the particulate layer (bottom layer) was further lysed with RIPA buffer before analyses. Another separation of the mitochondrial and cytosolic proteins was carried out according to a modified procedure of Adrain et al. [2001]. Briefly, cells were collected, washed with PBS and resuspended in cytosolic lysis buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.2, 200 μg/ml digitonin, 100 mM PMSF, protease inhibitor cocktail) for 5 min on ice. Cells were centrifuged at 1,000*g* for 5 min. The supernatant was kept as the cytosolic fraction. The pellet was washed in the cytosolic extraction buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES pH 7.5) and centrifuged at 11,000*g* for 10 min twice. The washed pellet was then digested in 50 μg/ml Proteinase K, 10 mM $CaCl_2$, 50 mM Tris–HCl, pH 8.0 for 15 min on ice. The digestion was stopped by adding 2 mM EDTA, 2 mM EGTA, 1 mg/ml BSA, 100 μM PMSF in 50 mM Tris–HCl pH 8.0. The sample was centrifuged at 11,000*g* for 10 min. The digested pellet was resuspended in two volumes of mitochondrial lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, PMSF, protease inhibitor cocktail) for 5 min on ice. The resulting suspension was centrifuged at 11,000*g* for 10 min to obtain the supernatant as the mitochondrial fraction.

Protein concentration of the preparation was determined by the bicinchoninic acid method [Smith et al., 1985]. Forty micrograms of protein samples were analyzed by SDS–polyacrylamide gel electrophoresis. The separated proteins were blotted onto polyvinylidene difluoride membranes (Immobilon, Millipore). The target proteins were detected by rabbit anti-antiquitin antibody (Epitomics); mouse anti-CoxIV antibody (Abcam), mouse anti-tubulin antibody (Santa Cruz), rabbit anti-PMP70 (peroxisomal membrane protein) (Abcam), rat anti-Grp94 (glucose-regulated protein) (Assay Designs), and rabbit anti-LAMP2B (lysosome-associated membrane glycoprotein 2 precursor) (Abcam) antibodies, followed by horseradish peroxidase-conjugated anti-rabbit (GE Healthcare), anti-mouse IgG (GE Healthcare) or anti-rat (Invitrogen) IgG secondary antibody with ECL Western blotting reagents (GE Healthcare).

FLOW CYTOMETRIC ANALYSIS OF ANTIQUITIN IN MITOCHONDRIA

HEK293 cells were trypsinized and centrifuged at 600*g* for 5 min. They were washed and counted in ice-cold PBS. Two milliliters of extraction buffer (10 mM HEPES, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, pH 7.5) with 2 mg/ml BSA was added to 50 million cells. After incubating on ice for 15 min, the cells were homogenized using a Dounce homogenizer (27 strokes) to get 60% breakage, as estimated by staining aliquot with trypan blue. The homogenate was centrifuged at 600*g* for 5 min to obtain the supernatant (intact mitochondria) which was centrifuged again at 11,000*g* for 10 min. The pellet was digested in the aforementioned buffer containing 50 μg/ml Proteinase K for 15 min on ice. The digestion was stopped by adding 2 mM EDTA, 2 mM EGTA, 1 mg/ml BSA, 100 μM PMSF in 50 mM Tris–HCl pH 8.0. The sample was centrifuged at 11,000*g* for 10 min. The digested pellet was resuspended in PBS as the mitochondrial preparation. The integrity of the isolated mitochondria was checked by JC-1 (Invitrogen) uptake according to the manufacturer's protocol. Fifty nanomolar of valinomycin was included as a positive control. To test for the presence of antiquitin, aliquots of mitochondria were fixed with ice cold 70% ethanol overnight at 4°C. They were then incubated overnight with the different primary antibodies (1:100) in the aforementioned high potassium buffer at 4°C. After rinsing with buffer, the preparations were incubated with the corresponding FITC-conjugated secondary antibody for 2 h at 4°C before analyses in a FACSCanto flow cytometer equipped with an argon laser and a CellQuest software (Becton Dickinson). Ten thousand events were acquired from each sample. The populations were identified by their light-scattering characteristics, enclosed in electronic gates, and analyzed for the intensity of the fluorescent probe signal.

RESULTS

PRESENCE OF A SIGNAL PEPTIDE AT THE NEWLY IDENTIFIED N-TERMINAL OF HUMAN ANTIQUITIN

Human antiquitin was once generally believed to be a cytosolic aldehyde dehydrogenase with 511 amino acids. However, a recent analysis of the nucleotide sequence of human antiquitin indicates another upstream potential start codon which, if used in initiating the translation process, will lead to an additional 28 amino acid peptide fragment (SP) at the N-terminal of the protein (sequence updated in the NCBI GenBank, May 2008) (Fig. 1). In silico analysis of SP by WoLF PSORT (http://wolfpsort.org/) showed that it encoded a mitochondrial targeting signal. Comparison of SP with the N-terminal of other mitochondrial proteins, for example, ALDH2, CoxIV and cytochrome *c*, also shows a high sequence consensus (Table II).

EXPERIMENTAL EVIDENCE FOR THE MITOCHONDRIAL TARGETING PROPERTY OF THE SIGNAL PEPTIDE

Expression of fusion proteins with EGFP was employed to study the subcellular localization of human antiquitin. Using the pEGFP.N1 vector, antiquitin was fused to the N-terminal of EGFP and examined under a confocal microscope. As a control, when only the

TABLE II. Sequence Alignment of the Putative Mitochondrial Targeting Signal Peptide of Human Antiquitin (ALDH7A1) With Those of Known Mitochondrial Proteins

GenBank accession no.	Protein name	Position	Amino acid sequence
NP_001173.2	ALDH7A1	1–28	MWRLPRALCVHAAKTSKLSGPWSRPAAF
NP_000681.2	ALDH2 precursor	1–17	MLRAAARFGPRLGRRLLS
NP_001852.1	CoxIV	1–30	MLATRVFSLVGKRAISTSVCVRAHESVVKS
NP_061820.1	Cytochrome *c*	1–30	MGDVEKGKKIFIMKCSQCHTVEKGGKHKTG

The positively charged amino acid residues which might lead to mitochondrial targeting of the protein are shown in bold.

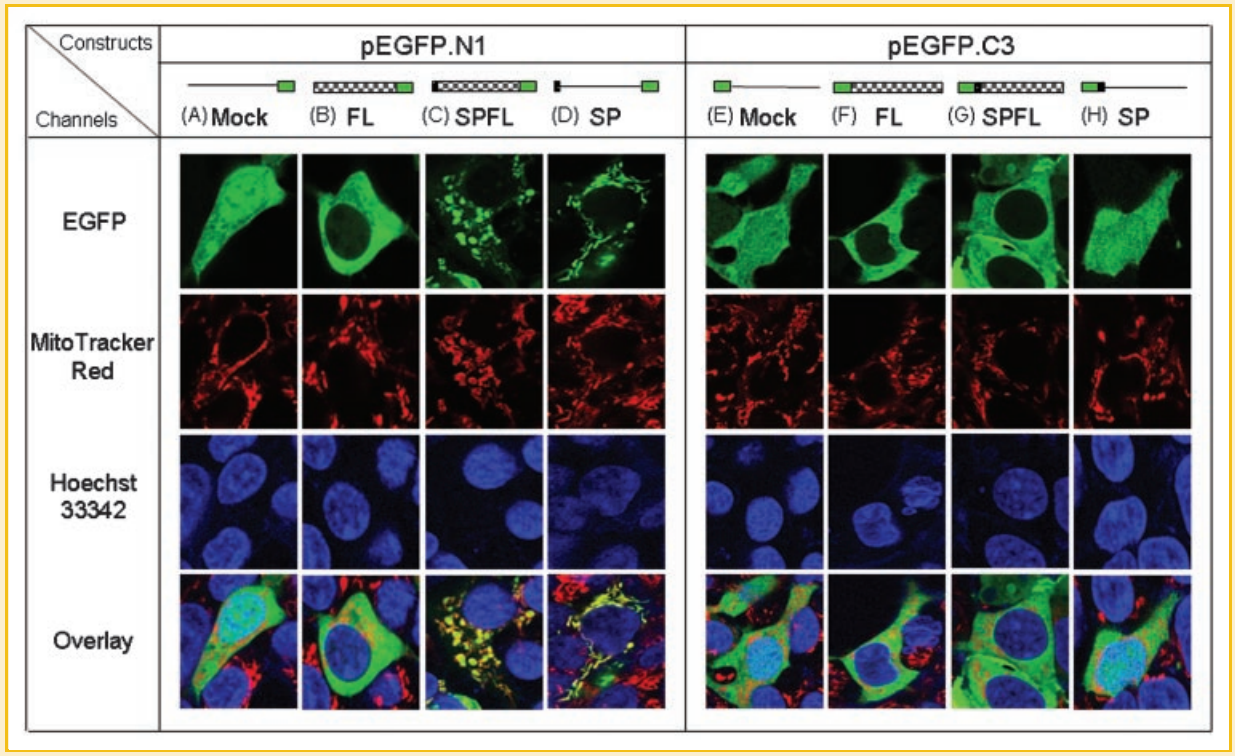

Fig. 2. Confocal microscopy of HEK293 cells expressing different antiquitin-EGFP fusion constructs. Different constructs were transiently transfected into HEK293 cells using Lipofectamine for 48 h. (A) pEGFP.N1 vector only, (B) FL-EGFP, (C) SPFL-EGFP, (D) SP-EGFP, (E) pEGFP.C3 vector only, (F) EGFP-FL, (G) EGFP-SPFL, and (H) EGFP-SP. Mitochondria and nuclei were stained with 500 nM of MitoTracker Red CMX-Ros and 1 mM Hoechst33342, respectively. The fluorescence signals were obtained by confocal microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

vector was transfected, a green fluorescent signal was observed in all the different compartments of the cells, including cytosol and nucleus (Fig. 2A). However, when the construct FL-EGFP was used, the fluorescence signal could only be detected in the cytosol and failed to show any overlap with that of the MitoTracker (Fig. 2B). The subcellular localization of antiquitin was different when the upstream 28 amino acids (signal peptide, SP) were added to FL to form the construct SPFL-EGFP. The fluorescent signal was found not only in the cytosol, but also in the mitochondria as the EGFP signal showed a partial overlap with that of the MitoTracker (Fig. 2C). To investigate whether SP itself possesses the mitochondrial targeting signal, the simple SP-EGFP construct was used. Confocal analysis showed a complete overlap between the fluorescent signals of EGFP and the MitoTracker (Fig. 2D). In a parallel set of experiments, FL, SPFL and SP were fused to the C-terminal of EGFP using the pEGFP.C3 vector. Under such conditions, SP lost its mitochondrial targeting property (Fig. 2G,H).

PRESENCE OF ANTIQUITIN IN BOTH MITOCHONDRIA AND CYTOSOL

The subcellular localization of human antiquitin was also studied by direct analysis of the HEK293 cells using an immunofluorescence technique. The staining pattern of antiquitin was similar to that of CoxIV, a well known mitochondrial marker protein. Both of them could overlap with the signal of the MitoTracker. They differ, however, in that the fluorescence signal could also be detected in the cytosol for antiquitin, but not for CoxIV (Fig. 3).

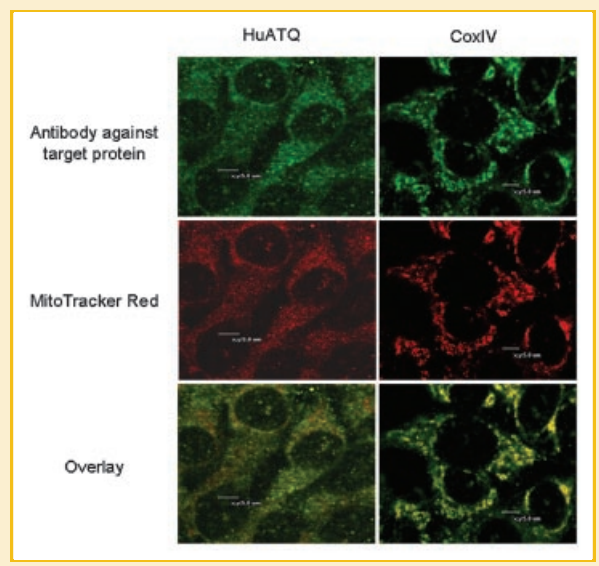

Fig. 3. Immunofluorescence analysis of human antiquitin localization in HEK293 cells. HEK293 cells were stained with (A) anti-antiquitin antibody, and (B) anti-CoxIV antibody, followed by the corresponding secondary antibody labeled with FITC or Alexa Fluor 488. Mitochondria were stained with 500 nM of MitoTracker Red CMX-Ros. The fluorescence signals were obtained by confocal microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

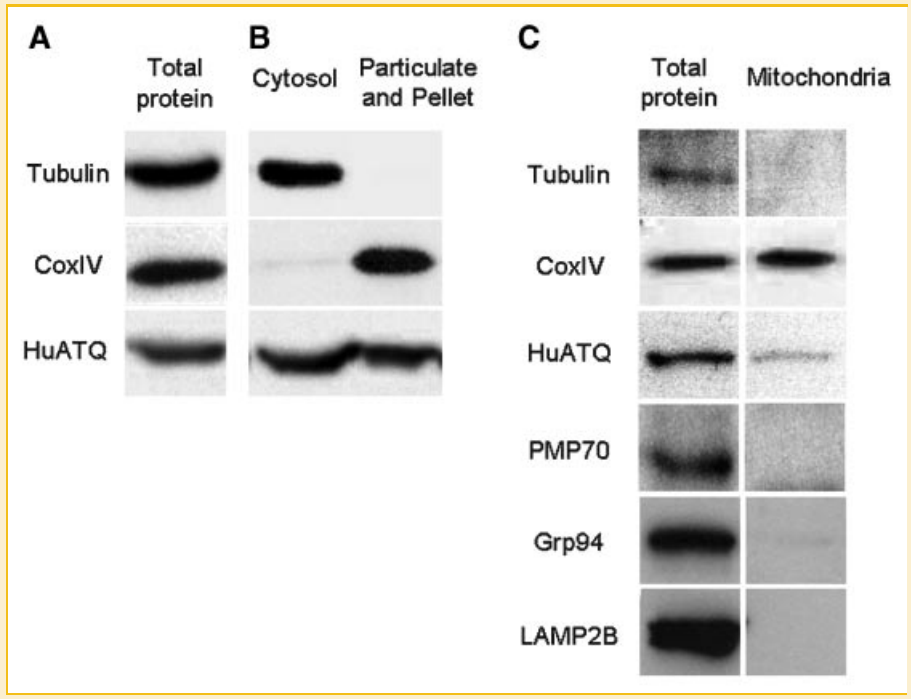

Fig. 4. Western blot analysis of human antiquitin in different subcellular fractions of HEK293 cells. (A) Total protein, (B) cytosolic and particulate protein obtained by BioVision Cytosol/Particulate Rapid Separation Kit, and (C) cytosolic and mitochondrial proteins obtained using the modified protocol of Adrain et al. [2001]. The proteins were resolved by SDS–polyacrylamide gel electrophoresis before Western blot analysis. Tubulin, CoxIV, PMP70, Grp94 and LAMP2B were used as marker proteins for cytosol, mitochondria, peroxisome, endoplasmic reticulum and lysosome, respectively.

To confirm the presence of antiquitin in both the cytosol as well as the mitochondria, the two subcellular fractions were isolated by two different methods for Western blot analyses. The enriched fractions obtained in both cases were free of cross-contaminations, as the cytosolic marker protein tubulin could only be detected in the cytosol, whereas the mitochondria marker protein CoxIV could be detected only in the particulate/mitochondria fraction (Fig. 4B). For human antiquitin, intense bands were observed in both the cytosol as well as the particulate/mitochondria fraction (Fig. 4C). The mitochondrial preparation was also free of contamination from other subcellular organelles, as no PMP70, Grp94 and LAMP2B, markers for peroxisome, endoplasmic reticulum and lysosome respectively, could be detected upon Western blot analysis (Fig. 4C).

Intact mitochondria were isolated for flow cytometric analysis. The integrity of the mitochondria preparation was shown by JC-1 staining and membrane depolarization could be observed after treatment with valinomycin (Fig. 5A). The fluorescent signal obtained after staining for antiquitin was higher than those for the peroxisome, endoplasmic reticulum and lysosome markers, albeit lower than that for CoxIV, the mitochondrial marker (Fig. 5B).

DISCUSSION

Subcellular localization of proteins can be studied by both in silico and in vitro methods. Sequence analysis of human antiquitin (ALDH7A1) showed the presence of a large hydrophobic region between amino acid residues 155 and 208. Such region is also present in its garden pea counterpart ALDH7B1. This region was once suggested to be a transmembrane segment of the protein [Lee et al., 1994]. Subsequent analysis showed that this region can also be found in other members of the ALDH superfamily. Crystal structures of human antiquitin (PDB:2J6L), seabream antiquitin [Tang et al., 2008] and others ALDHs [Steinmetz et al., 1997; Johansson et al., 1998; Moore et al., 1998] showed that this region is actually part of the NAD^+-binding domain. The hydrophobicity probably contributes towards NAD^+ binding rather than serves as a transmembrane segment.

In silico analysis of the entire amino acid sequence can also be used to study the subcellular localization of a protein. Based on the sequence alignment with other ALDHs, human antiquitin was once generally believed to be an enzyme of 511 amino acids. Analysis of this sequence (FL) by the WoLF PSORT program (http://wolfpsort.org/); [Horton et al., 2007] fails to indicate any significant subcellular localization signals; instead, it is most likely to be a cytosolic protein. However, a more detail analysis of the nucleotide sequence indicates another upstream potential start codon which might lead to an additional 28 amino acid peptide fragment at the N-terminal of the protein. It is possible that this peptide (SP) is involved in subcellular targeting. In silico analysis by WoLF PSORT shows that SP does encode a targeting signal which could direct the protein into mitochondria. The mitochondrial targeting signal peptide is usually found at the N-terminal, and is characterized by enriched positively charged residues, lack of negatively charged residues, and ability to form amphiphilic α-helix.

To obtain experimental proof of such prediction, expression of fusion proteins with EGFP was used. Consistent with the

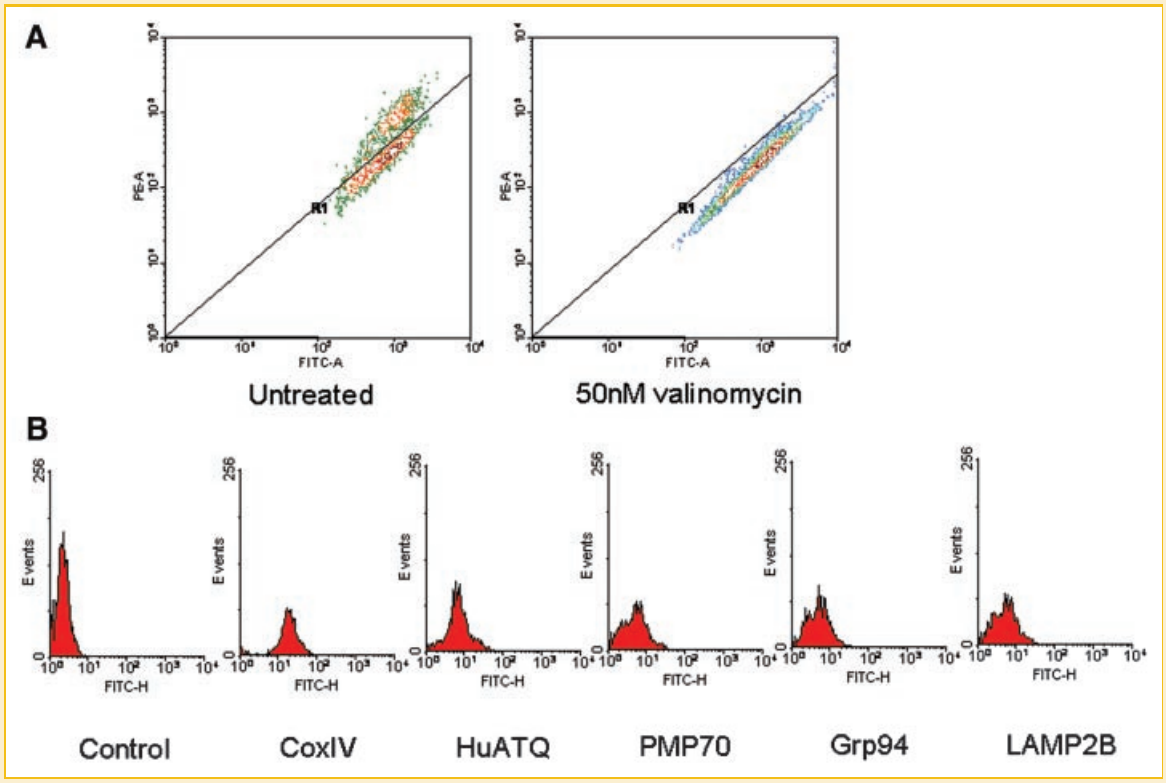

Fig. 5. Immunostaining and flow cytometric analysis of human antiquitin in isolated mitochondria from HEK293 cells. (A) JC-1 staining of isolated mitochondria before and after treatment with 50 nM valinomycin. (B) Immunostaining in isolated mitochondria from HEK293 cells. Labeling was carried out using primary antibody against antiquitin, CoxIV, PMP70, Grp94 or LAMP2B, followed by the corresponding FITC-conjugated secondary antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

computational analysis, SP-EGFP was expressed exclusively in the mitochondria whereas FL-EGFP was found only in the cytosol. Thus, the present experimental result indicates that the 28-amino acid SP does contain a mitochondrial targeting signal which might be critical for the exact subcellular localization of antiquitin, although it remains to be solved which start codon is the one actually used in the translation process. On the other hand, the SPFL-EGFP conjugate could be found in both mitochondria and cytosol. It remains elusive whether post-translational modifications, incomplete transportation of mitochondrial protein or retrograde transport of mitochondrial protein back to the cytosol is responsible for the presence of antiquitin in the cytosol [Regev-Rudzki and Pines, 2007].

Signal peptide can be present at the N- or C-terminal of a protein. For example, mitochondrial ALDH2 possesses an N-terminal mitochondrial targeting signal [Wang et al., 1989] whereas a microsomal ALDH is localized in the endoplasmic reticulum by virtue of a hydrophobic signal peptide of 35 amino acids at its C-terminus [Masaki et al., 1994]. Removal of this hydrophobic tail results in the cytosolic localization of the protein. In the present study, the pEGFP.C3 plasmid was included such that the presence of EGFP at the N-terminal of the protein will not mask the effect of the signal peptide at the C-terminal of the protein, if there is any. The results failed to indicate any specific targeting for all the different constructs, including EGFP-FL, EGFP-SPFL and EGFP-SP.

Using the EGFP system, seabream antiquitin was previously suggested to be a cytosolic protein. The FL-EGFP conjugate is found to be located in the cytosol but not the mitochondria in transiently transfected cell [Tang et al., 2005]. Similar to the case in the human enzyme, detail analysis of the 5′ RACE results of seabream antiquitin [Tang et al., 2005] indicates the presence of an additional potential start codon located 84 nucleotides upstream of the originally proposed start codon for FL antiquitin. Our unpublished data show that this segment codes for a signal peptide and directs the protein into the mitochondria where it is believed to be processed by certain peptidase to give a truncated, mature form of the protein. This may account for the short form of seabream antiquitin purified from the liver [Tang et al., 2002]. Thus, the original suggestion that seabream antiquitin is a cytosolic enzyme needs further confirmation.

Conjugation with EGFP is a common method to study the subcellular localization of protein. However, it is an artificial method and the results will be questionable when there is more than one potential start codon for the protein. To tackle this problem, the site of expression of human antiquitin is studied by the immunofluorescence method in which the cells were directly stained with antibody against antiquitin. Another approach used is the conventional method of physically separating the different organelles before analyses. The purity of the mitochondrial preparation was confirmed by using the various subcellular organelles markers. Using two different separating methods, human antiquitin was found to be present both in the cytosol and the mitochondria. In this regard, it is noted that a dual subcellular localization in the endoplasmic reticulum and peroxisomes has

TABLE III. Prediction of Subcellular Localization of Antiquitins From Different Species

	Protein	Predicted subcellular localization
Animal antiquitins		
Homo sapiens	NP_001173.2	Mitochondria, cytosol _mitochondria
Macaca mulatto	XP_001111886.1	Mitochondria, cytosol _mitochondria
	XP_001111963.1	Mitochondria, cytosol _mitochondria
	XP_001095681.1	Mitochondria, cytosol _mitochondria
	XP_001096111.1	Mitochondria, cytosol _mitochondria
	XP_001096223.1	Mitochondria, cytosol _mitochondria
Rattus norvegicus	XP_214535.3	Mitochondria, cytosol _mitochondria
Mus musculus	NP_613066.2	Plasma membrane, mitochondria
	NP_001120810.1	Plasma membrane, peroxisome
Acanthopagrus schlegelii	AAX54912.1	Mitochondria, cytosol _mitochondria
Danio rerio	AAI65754.1	Cytosol, cytosol_nucleus
	AAH44367.1	Mitochondria, cytosol_mitochondria
Plant antiquitins		
Arabidopsis thaliana	NP_849807.1	Cytosol, plasma membrane
	NP_175812.1	Cytosol, plasma membrane
Oryza sativa (japonica cultivar-group)	NP_001063281.1	Chloroplast, cytosol
Glycine max	AAP02957.1	Nucleus, cytosol
Malus x domestica	BAA75633.1	Cytoskeleton, cytosol
Pisum sativum	CAA38243.1	Cytosol, endoplasmic reticulum

The mRNA sequence of antiquitin from different species was translated by ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The subcellular localization was predicted by using the WoLF PSORT program (http://wolfpsort.org/).

recently been reported for fatty ALDH [Ashibe et al., 2007]. The subcellular localization of the enzyme is related to its physiological role. The demonstration of the presence of human antiquitin in mitochondria is consistent with its proposed physiological role in the oxidation of α-AASA, an intermediate in the lysine catabolic pathway, which has been shown to be present in mitochondria or peroxisomes, but not in the cytosol [Rao et al., 1993]. Proteomic studies have also demonstrated the presence of human antiquitin in placenta [Lescuyer et al., 2003] and heart [Gaucher et al., 2004] mitochondria.

To understand more about the subcellular localization of antiquitin, the nucleotide sequence of antiquitin from different species were compared. In view of the existence of possible upstream inframe translation start site, mRNA sequence of different antiquitins were translated by ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), followed by analyses using the subcellular localization prediction program WoLF PSORT. The results show that most animal antiquitins show a dual localization in mitochondria and cytosol while their plant counterparts are mostly cytosolic (Table III). A recent study using fluorescent microscopy provided direct experimental proof that rice antiquitin is a cytosolic enzyme [Shin et al., 2009]. In general, animal antiquitins differ from plant antiquitins in having an additional potential start codon upstream of the FL one. Initiation of translation at this additional start codon will lead to the synthesis of the signal peptide-bearing form of the enzyme and direct the protein into the mitochondria. With different subcellular localization, it is anticipated that antiquitins from the two kingdoms might not have identical physiological function.

To conclude, the present study, using three different experimental approaches, demonstrates that human antiquitin is present in both the mitochondria and cytosol. Human antiquitin is synthesized as a precursor form with the signal peptide SP which directs the protein into the mitochondria. At this stage, it is unclear whether the cytosolic antiquitin observed came from the retrograde transport [Singh and Gupta, 2006] of the mitochondrial antiquitin, or is synthesized by using the other start codon [Kozak, 1996]. Nevertheless, the demonstration of the presence of antiquitin in mitochondria is consistent with its proposed physiological role in lysine metabolism.

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