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Expression analysis of two mutant human ornithine transcarbamylases in COS-7 cells

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Abstract Ornithine transcarbamylase (OTC) is located in the mitochondrial matrix of the liver and small intestine and catalyzes the second step of the urea cycle. OTC deficiency (OTCD) is an X-linked inborn error of metabolism and causes hyperammonemia. We reported in 1992 the A152V and G195R mutations in patients with OTCD. These mutant OTC cDNAs were prepared by site-directed mutagenesis using the polymerase chain reaction (PCR). The wild-type and mutant cDNAs were transiently expressed in COS-7 cells. The wild-type cDNA gave an OTC activity of 1180±47 nmol/min per mg protein. The OTC activities of the A152V and the G195R mutants were 3.7% and 2.5% of that of wild-type, respectively. Immunoblot analysis showed that the quantities of OTC proteins in the A152V and G195R mutants were 29% and 12% of that of wild-type, respectively. In pulse-labeling and pulse-chase experiments, the precursor form of OTC was synthesized and processed to the mature form. The A152V mutant OTC was processed to the mature form as rapidly as the wild-type precursor. However, the processed, mature form of the mutant OTC was rapidly degraded, presumably in the mitochondrial matrix. These results indicate that OTCD with the A152V mutation is due both to rapid degradation of the processed, mature form, and to a lower specific activity of the remaining protein.

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Introduction

Ornithine transcarbamylase (OTC) is located in the mitochondrial matrix of the liver and catalyzes the second step of urea synthesis. OTC is initially synthesized as a larger precursor with the NH_2-terminal presequence (pOTC), and is then imported into the mitochondrial matrix and processed to the mature form (Mori et al. 1981; Conboy and Rosenberg 1981; Horwich et al. 1984; Takiguchi et al. 1984).

Ornithine transcarbamylase deficiency (OTCD) is X-linked and is the most common inborn error of the urea cycle. Biochemical studies showed that the disease results from either quantitative or qualitative changes of the enzyme (Satoh et al. 1992). About 90 different mutations associated with OTCD have been reported. They include point mutations, small deletions and insertions, and gross deletions (Brusilow and Horwich 1995). Expression analysis in mammalian cells has been performed for several cases of OTCD (Lee and Nussebaum 1989; Nishiyori et al. 1997; Matsuura et al. 1994), and the relationship between mutations and molecular pathology or clinical phenotypes is being analyzed. However, analysis of a possible defect in OTC import into mitochondria is lacking.

Recently, a procedure to analyze synthesis of pOTC, and its processing in cultured cells by short time pulse-labeling and pulse-chase experiments, was developed (Kanazawa et al. 1997; Terada et al. 1997). We analyzed two mutants using this pulse-chase procedure as well as the routine expression analysis in COS-7 cells. Here, we report that A152V and G195R mutations resulted in both decreased enzyme protein levels and lower specific activities of the remaining enzymes. We also report that the A152V mutant precursor was processed normally to the mature form and that the processed form was rapidly degraded, probably in the mitochondrial matrix.

Materials and methods

Plasmid construction

Plasmid pCAGGS/hOTC (Kanazawa et al. 1997) was constructed by inserting the wild-type human OTC cDNA into pCAGGS (Niwa et al. 1991) . Using this plasmid as a starting material, we constructed two kinds of plasmid carrying the mutant OTC cDNA sequences. To construct mutant expression vectors carrying the A152V and G195R mutations, pCAGGS/hOTC was digested with *Hin*dIII and the first polymerase chain reaction (PCR) was performed using the four primers (OTC-292-U 5'-CCCCCGCTGGCTAACTTGCTGT-3', OTC-C454T-L 5'-TGCTTCTTTAACCAGGGTGTCCAAAT-3' and OTC-C454T-U 5'- TTTGGACACCCTGGTTAAAGAAGCATC-3', OTC-1109-L 5'-ATTGCCTCCATGCGCTGCTTCCAAT-3') and (OTC-292-U, OTC-G538A-L 5'-TTCCCATCCCTGATCCAGCTGA-3' and OTC-G538A-U 5'-TCAGCTGGATCAGGGATGGGAAC-3', OTC-1109-L). Substitutions in the primers are underlined. Next, each product was amplified using the two primers OTC-292-U and OTC-1109-L. The PCR reaction was performed in a final volume of 100 µl containing 0.5 µg of template, 250 µM dNTP mix, 1 × Expand High-Fidelity buffer 2, 3.5 U of Expand High-Fidelity (Boehringer Mannheim, Mannheim, Germany), and 50 nmol of each primer. The PCR parameters were: 94°C for the first 2 min for denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1.5 min, and extension at 65°C for 1 min.

Then, pCAGGS/hOTC and the second set of PCR products were digested with *Csp*45I/*Kpn*I. To express mutant human OTCs in COS-7 cells, the 817-bp *Csp*45I and *Kpn*I fragments prepared from pCAGGS/hOTC and the second set of PCR products were ligated with T4 DNA ligase. Plasmids were amplified in BMH71-18, and were then purified by the plasmid purification kit (Qiagen, Hilden, Germany).

Determination of nucleotide sequence

Determination of the nucleotide sequence was performed using Thermosequenase (Amersham, Tokyo, Japan) or the DSQ1000L (Shimazu, Kyoto, Japan).

DNA transfection

COS-7 is an immortalized African green monkey cell line. The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and maintained in a 5% CO_2 atmosphere at 37°C. Transfection of DNA was performed by *Trans* IT™-LT1 (Takara, Kyoto, Japan). Cells (5×10^5) in 10-cm culture dishes were transfected with 8 µg of plasmid DNA and 2 µg of pAc-lacZ (Miyazaki et al. 1989). Cells were incubated with plasmids for 4 h in a 5% CO_2 atmosphere at 37°C, then washed with DMEM, further incubated in DMEM containing 10% FCS for 24 h and harvested with trypsinization. The cells were stored at −80°C until analysis.

Enzyme and protein assays

After transient expression, the cells were harvested with trypsinization and washed with phosphate-buffered saline (PBS), and then divided into halves. One half was suspended in extraction buffer [20 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 20% glycerol, 1% Triton X-100, and 1 mM dithiothreitol (DTT) at pH 7.4], disrupted by freezing and thawing, and then centrifuged at 14,000 *g* for 10 min for assay of OTC. The cell extracts were used for protein assay, OTC assay, and immunoblot analysis.

The other half was used for assay of β-galactosidase using the β-galactosidase enzyme assay system (Promega, Madison, WI, USA).

OTC activity was measured by the colorimetric method of Brown and Cohen (1959) and was normalized for β-galactosidase activity where indicated. Protein was measured using the DC protein kit (Bio-Rad Laboratories, Richmond, CA, USA).

Electrophoresis and immunoblots

Polyacrylamide gel (10%) electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed by the method of Laemmli (1970). Protein was transferred to a nitrocellulose membrane, and the membrane was incubated with the OTC antiserum (1:1000 dilution) (Kanazawa et al. 1997). OTC protein was then detected with the ABC-PO kit (Vector, Burlingame, CA, USA) and the ECL kit (Amersham) and determined densitometrically.

Pulse-labeling and pulse-chase experiments

COS-7 cells were transfected with 20 µg of expression plasmids. After 24 h, the cells were harvested with trypsinization, washed twice with PBS and suspended in 3 ml of methionine-free DMEM. The suspended cells were preincubated at 37°C for 1 h in a 50-ml conical tube and radiolabeled with 8 MBq of Pro-mix containing L-[^{35}S]-methionine and L-[^{35}S]-cysteine (Amersham) for the indicated periods. Aliquots (0.6 ml) were removed at the indicated times and mixed with 0.4 ml of ice-cold 25 mM Tris-HCl (pH 7.4) containing 5 mM ethylenediaminetetraacetic acid (EDTA), 0.25% SDS, 0.25% Triton X-100, 125 µM chymostatin, 125 µM leupeptin, and 125 µM antipain. The cell lysates were clarified by centrifugation at 10,000 *g* for 10 min. Radiolabeled proteins were immunoprecipitated, subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography, and quantified by imaging plate analysis using a FUJIX BAS2000 analyzer (Fujifilm, Tokyo, Japan).

In pulse-chase experiments, the cells were suspended in 1 ml of methionine-free medium, preincubated for 1 h, radiolabeled for 5 min, and then chased by adding 2 ml of medium containing 20 mM methionine.

Results and discussion

Expression of mutant enzymes in COS-7 cells

Expression plasmids encoding two mutant pOTCs (A152V and G195R) were constructed by site-directed mutagenesis using PCR. The mutations were confirmed by sequencing. The plasmids encoding wild-type pOTC and mutant pOTCs were transfected into COS-7 cells, and OTC activity in COS-7 cells was measured. Nontransfected COS-7 cells or cells transfected with the control plasmid pCAGGS had no detectable OTC activity. When the plasmid for wild-type pOTC was transfected, a high OTC activity (1180±47 nmol/min per mg protein) was detected. Enzyme activities (normalized for β-galactosidase activity) in the cells transfected with plasmids for the mutant pOTCs A152V and G195R were 3.7% and 2.5% of the control value, respectively.

The OTC protein was studied by immunoblot analysis. The plasmid encoding wild-type pOTC gave an immunoreactive polypeptide of 36 kDa (Fig. 1). This polypeptide was absent in nontransfected cells. The amounts of OTC protein (normalized for β-galactosidase activity) in COS-7 cells expressing A152V pOTC and G195R pOTC were 29% and 12% of control, respectively. The OTC activity and protein agree with those in liver samples from patients (Table 1). These results indicate that OTCDs with A152V and G195R mutations are due both to decreased enzyme protein levels and to decreased specific activities of the remaining enzyme.

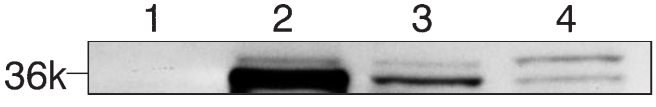

Fig. 1 Immunoblot analysis of wild-type and mutant ornithine transcarbamylases (OTCs) in transfected COS-7 cells. COS-7 cells were transfected with 2 μg each of pAc-lacZ and 8 μg each of pCAGGS *(lane 1)*, pCAGGS/hOTC (wild-type) *(lane 2)*, that encoding A152V *(lane 3)*, or that encoding G195R *(lane 4)*. Cells extracts (10 μg of protein) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were electrotransferred to a nitrocellulose membrane

Synthesis and mitochondrial import and processing of wild-type and A152V pOTCs

We previously performed cell-free synthesis of pOTC using liver RNAs from a control and from patients with the A152V mutation, and showed that OTC mRNA activity in the patient was similar to that of the control (Satoh et al. 1992). Therefore, we speculated that the decreased OTC protein in the patient is due either to impaired import of pOTC into the mitochondria or to enhanced degradation of imported enzyme protein. To test these possibilities, we performed pulse-labeling experiments.

When the COS-7 cells expressing wild-type pOTC were labeled with [^{35}S]methionine, labeled pOTC appeared by 1 min and increased gradually with time up to 16 min (Fig. 2). On the other hand, processed, mature OTC appeared with a lag time of about 4 min and then increased rapidly. Processing of pOTC reflects its import into the mitochondria, since it occurs in the mitochondrial matrix during or immediately after the mitochondrial import (Mori et al. 1981; Conboy and Rosenberg 1981). When the cells expressing A152V pOTC were labeled, labeled mutant pOTC was synthesized as rapidly as wild-type pOTC, but its increase was less than that of the wild-type. Processed mutant OTC appeared at 4 min, but its increase was much less than that of the wild-type.

We then performed pulse-chase experiments (Fig. 3). The cells expressing wild-type or mutant pOTC were labeled with [^{35}S]methionine for 5 min and then chased by cold methionine for various periods. In the case of the wild-type, the labeled pOTC decreased in the chase and the mature OTC increased concomitantly. In contrast, in the case of the A152V mutant, labeled pOTC decreased with time, and mature OTC decreased slowly. All these results indicate that wild-type pOTC was efficiently imported into the mitochondria and processed to the mature form, and that the mature form remained stable under the conditions. The mutant A152V pOTC was also imported and processed efficiently, but the processed, mature form was degraded rapidly, presumably in the mitochondrial matrix, leading to a decreased enzyme protein level.

Table 1 Ornithine transcarbamylase (OTC) activity and protein in patients' liver and in COS-7 cells expressing mutant OTCs

Sample		Wild-type	A152V	G195R
Liver[a]	OTC activity (%)	100	10	6
	OTC protein (%)		Decreased	Markedly decreased
COS-7 cells	OTC activity (%)[b]	100 ± 10.3	3.7 ± 1.22	2.5 ± 0.83
	OTC protein (%)[b]	100 ± 7	29 ± 3	12±2

[a] The results for liver samples are from Satoh et al. (1992).
[b] OTC activity and protein were normalized for β-galactosidase activity and are shown as mean ± SD (n = 3). The activity with wild-type OTC cDNA was 1180 ± 47 nmol/min per mg protein (100% ± 10.3%) (n = 3).

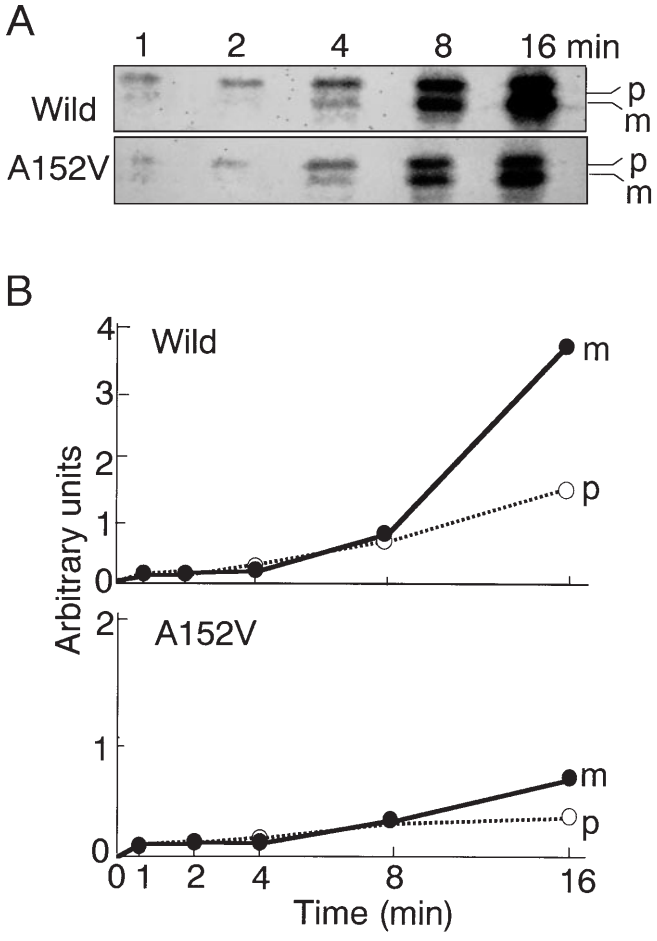

Fig. 2 Pulse-labeling experiments. COS-7 cells were transfected with 20 μg each of pCAGGS/hOTC or pCAGGS/hOTC(A152V). After 24 h, the cells were radiolabeled. Radiolabeled OTC and its precursor (pOTC) were immunoprecipitated with 20 μl of anti-OTC serum and 200 μl of a 10% suspension (v/v) of protein A-Sepharose, and subjected to 10% SDS-PAGE and fluorography. Radioactive polypeptides were visualized (**A**) and quantitated (**B**) by imaging plate analysis. *p* and *m*, precursor and mature form of OTC

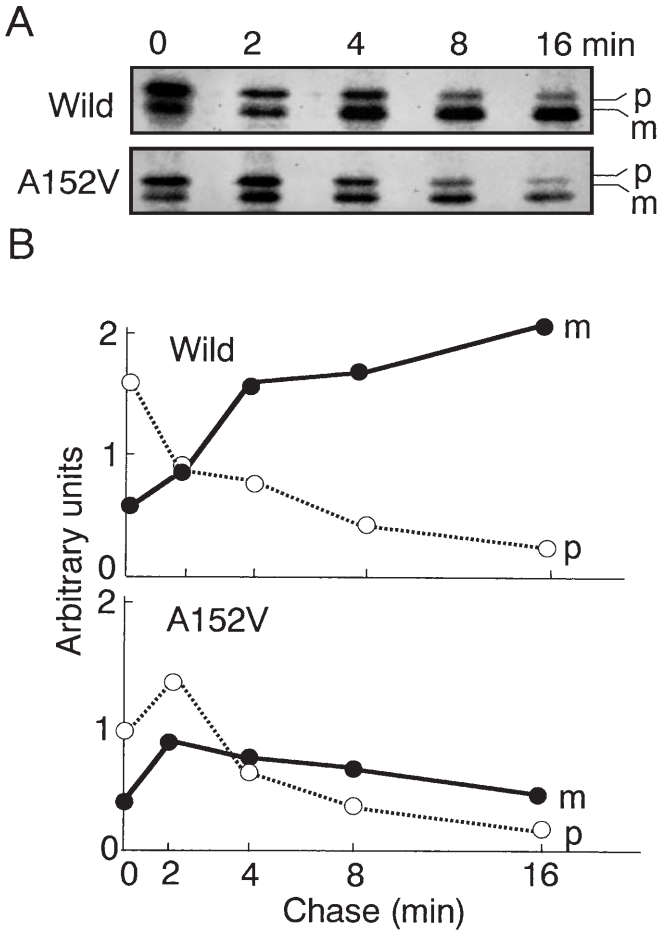

Fig. 3 Pulse-chase experiments. COS-7 cells were transfected with 20 μg each of pCAGGS/hOTC or pCAGGS/hOTC(A152V). After 24 h, the cells were labeled for 5 min and then chased for the indicated periods. Immunoprecipitation, SDS-PAGE, fluorography, and imaging plate analysis were performed as described in Fig. 2

It is speculated that some cases of deficiencies of mitochondrial proteins, including OTCD, may be due to impaired import of the corresponding precursor proteins (Isaya et al. 1988). Mitochondrial protein import and processing are very rapid (Mori et al. 1981) and cannot be analyzed by routine pulse-chase experiments lasting several hours. In the present study, we report the first analysis of an OTC mutation by using a rapid pulse-chase procedure lasting for a few minutes. This method will be useful to analyze possible defects in mitochondrial import and processing of precursor proteins in mitochondrial diseases.

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