Instructions for use

Title
OCTN2VT, a splice variant of OCTN2, does not transport carnitine because of the retention in the endoplasmic reticulum caused by insertion of 24 amino acids in the first extracellular loop of OCTN2

Author(s)
Maekawa, Satoshi; Mori, Daisuke; Nishiya, Tadashi; Takikawa, Osamu; Horinouchi, Takahiro; Nishimoto, Arata; Kajita, Emi; Miwa, Soichi

Citation
Biochimica et Biophysica Acta (BBA) : Molecular Cell Research, 1773(6), 1000-1006
https://doi.org/10.1016/j.bbamcr.2007.04.005

Issue Date
2007-06

Doc URL
http://hdl.handle.net/2115/28237

Type
article (author version)

File Information
BBAM1773-6.pdf
OCTN2VT, a splice variant of OCTN2, does not transport carnitine because of the retention in the endoplasmic reticulum caused by insertion of 24 amino acids in the first extracellular loop of OCTN2

Satoshi Maekawa\textsuperscript{a,1}, Daisuke Mori\textsuperscript{a,1}, Tadashi Nishiya\textsuperscript{a,1}, Osamu Takikawa\textsuperscript{b}, Takahiro Horinouchi\textsuperscript{a}, Arata Nishimoto\textsuperscript{a}, Emi Kajita\textsuperscript{a}, and Soichi Miwa\textsuperscript{a,*}

\textsuperscript{a}Department of Pharmacology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan, \textsuperscript{b}Laboratory of Radiation Safety, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi 4740-8522, Japan

*Corresponding author: Soichi Miwa, Department of Pharmacology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan, Phone: +81-11-706-6919, Fax: +81-11-706-7824, Email: smiwa@med.hokudai.ac.jp (S.Miwa)

\textsuperscript{1}These authors contributed equally to this work.
Abstract

A novel organic cation transporter OCTN2 is indispensable for carnitine transport across plasma membrane and subsequent fatty acid metabolism in the mitochondria. Here, we report a novel splice variant of OCTN2 (OCTN2VT), in which a 72 base-pair sequence located in the first intron of OCTN2 gene was spliced between exons 1 and 2 of OCTN2, causing the insertion of 24 amino acids in the first extracellular loop of OCTN2. Despite the similarity between OCTN2 and OCTN2VT regarding primary structure and tissue distribution, their biochemical characteristics were significantly different. OCTN2 was expressed on the plasma membrane with robust N-glycosylation, whereas OCTN2VT was retained in the endoplasmic reticulum (ER) with poor N-glycosylation. In addition, the retention in the ER caused no carnitine uptake into the cells. These results demonstrate that the biochemical and functional characteristics of OCTN2VT are distinct from OCTN2 due to the insertion of 24 amino acids in the first extracellular loop.

Keywords: OCTN2; OCTN2VT; splicing; carnitine; N-glycosylation; endoplasmic reticulum
1. Introduction

Carnitine (β-hydroxy-γ-trimethylamino butyrate) is essential for transfer of long chain fatty acids from the cytosol to the mitochondrial matrix for subsequent β-oxidation to generate cellular energy [1]. The carnitine transport across the plasma membrane is mediated by novel organic cation transporter (OCTN) family proteins [2]. Three different OCTNs (OCTN1, OCTN2, and OCTN3) have been identified in humans and mice [3] and [4]. Among the OCTN family, OCTN2 has been shown to transport carnitine most efficiently, and malfunction of OCTN2 causes the primary systemic carnitine deficiency characterized by progressive cardiomyopathy, skeletal muscle myopathy, and hypoglycemia in humans [5]. The mice having a point mutation in OCTN2 have severe disturbance of energy generation, causing serious symptoms in the heart and skeletal muscles, in which fatty acids are a primary source for energy [6]. Therefore, OCTN2 is indispensable for energy homeostasis in the body.

The biochemical properties and physiological functions of OCTN2 have been well-established. OCTN2 transports carnitine in a Na\(^{+}\)-dependent manner [7]. OCTN2 is a twelve membrane-spanning protein with three putative N-glycosylation sites (Asn-57, -64, and -91) within the first extracellular loop [2]. Those sites are conserved in all members of an OCTN family [8], suggesting that OCTN2 enters the secretory pathway including endoplasmic reticulum (ER) and Golgi apparatus to be expressed on the plasma membrane.

Recently, we identified a novel up-regulator of OCTN2, cartregulin, in the rat brain [9]. Cartregulin is homologous to OCTN2, and upregulates the expression level of OCTN2 by stabilizing the mRNA of OCTN2. We also found a novel gene homologous to
OCTN2 by searching the human genome database “the Assembled EST (AssEST)”. This gene encodes a full-length OCTN2 with the insertion of 24 amino acids (QDSGAYNAMKNRMGKKPALCLPAQ) between Glu-131 and Trp-132 in the first extracellular loop of OCTN2. This variant type of OCTN2 (designated as OCTN2VT) was expected to possess the biochemical and physiological characteristics similar to the OCTN2, because of the similarity between OCTN2 and OCTN2VT regarding the primary structure. However, the actual characteristics of OCTN2VT and the mechanism by which OCTN2VT is produced are unknown.

In the present study, we attempted to elucidate these issues by using Hela cells stably expressing the yellow fluorescent protein (YFP)-tagged OCTN2VT. We found that despite the similar expression profiles in the human tissues between OCTN2 and OCTN2VT, their biochemical characteristics were significantly different. That is, (1) OCTN2 was highly N-glycosylated and expressed on the cell surface, whereas OCTN2VT was retained in the ER without N-glycosylation; (2) OCTN2VT-expressed HeLa cells could not take up carnitine due to the absence of OCTN2VT on the plasma membrane; (3) the DNA sequence encoding the 24 amino acids inserted in the OCTN2VT was located in the first intron of OCTN2 genome with being interposed between the GU-AG intron termini, suggesting the mechanism of the splicing to generate the OCTN2VT protein. Taken together, these results demonstrate that OCTN2VT, a novel splice variant of OCTN2, is biochemically and functionally distinct from OCTN2 and other OCTN family proteins.
2. Materials and Methods

2.1. Reagents and cell culture

Anti-GFP antibody (A.v. monoclonal antibody; clone JL-8) and anti-KDEL antibody were purchased from Clontech (Mountain View, CA) and Stressgen (Ann Arbor, MI), respectively. Peptide N-Glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA). L-[methyl-\(^3\)H]Carnitine HCl was purchased from Amersham Biosciences (Sydney, Australia). HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

2.2. PCR analysis

PCR was carried out by using human kidney cDNA library (Stratagene, La Jolla, CA) as a template for 35 cycles (94 °C for 15 s, 55 °C for 30 s, and 72 °C for 2 min). Four types of primer designated primer a, b, c, and d were used (Fig. 1C). Primer a (5’-CATGCGGGAGTACGACGAGGTGACC-3’) and primer d (5’-TCTTTTCCTCTCAGTTTCTCCTTA-3’) correspond to 5’- and 3’-ends of OCTN2, respectively. Primer b (5’-GGCTTTCTTTCCCATCCTGGTTTTTCA-3’) and primer c (5’-TACAATGCTATGAAAAACAGGATGG-3’) are reverse and forward primers, respectively, corresponding to 3’- and 5’-ends of the insert specific for OCTN2VT. The PCR products were subjected to gel electrophoresis to determine the sizes of the products.

2.3. Northern blot analysis

Northern blot analysis was done using MTN Multiple Tissue Northern Blot (Human
12-Lane, Clontech, Mountain View, CA) as described previously [9]. A 72 bp fragment specific for OCTN2VT was used as a probe.

2.4. DNA constructs

The cDNAs encoding human OCTN2 and OCTN2VT were amplified by PCR from the human kidney cDNA library. The N91Q mutation was introduced by PCR-based site directed mutagenesis. Primers used here were 5’-CCACCATCGCCAGTTCTCGCGCT-3’ and 5’-AGCGCCGAGAACTGGCGATGGTGG-3’). The retroviral vectors for YFP-tagged OCTN2, OCTN2VT, and OCTN2(N91Q) were constructed as described previously [10].

2.5. Analysis of expression level by flow cytometry and immunoblotting

Introduction of OCTN2YFP, OCTN2VTYFP, and OCTN2(N91Q)YFP into HeLa cells was carried out by retroviral gene transfer as described previously [11]. The cells stably expressing OCTN2YFP, OCTN2VTYFP, or OCTN2(N91Q)YFP were selected by 2 μg/ml puromycin for three days. The expression levels of OCTN2YFP, OCTN2VTYFP, and OCTN2(N91Q)YFP were determined by flow cytometric analysis as described previously [11]. More than 95% of the cells expressed those proteins. The whole cell lysates were prepared as described previously [12]. All samples were loaded on gels without being boiled, because OCTN2 and OCTN2VT were highly aggregated by the sample-boiling step. The membrane fractions were prepared as described in Takaesu et al [13]. The protein concentration of each fraction was measured with the Bio-Rad Protein Assay kit (Bio-Rad,
Hercules, CA). Equal amounts of protein were loaded in each lane and separated by SDS-polyacrylamide gel electrophoresis. Gels were transferred onto Immobilon-P membranes (Millipore, Bedford, MA), and blotted with anti-GFP antibody. In the case of PNGase F treatment, 3 μg of OCTN2 membrane fraction and 50 μg of OCTN2VT membrane fraction were treated with PNGase F for 1 h at 37 °C. Then, the whole proteins were loaded and separated by SDS-PAGE. In the case of MG132 treatment, HeLa cells retrovirally expressing OCTN2VT were treated with 10 μM MG132 for indicated periods, and then the whole cell lysates were prepared.

2.6. Microscopy

HeLa cells expressing OCTN2YFP or OCTN2VTYFP were plated onto a glass bottom dish (IWAKI, Japan). Next day, the cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen, San Diego, CA). Then, the cells were treated with anti-KDEL antibody followed by Alexa Fluor 594-conjugated anti-mouse IgG antibody. After washes, subcellular distributions of OCTN2YFP and OCTN2VTYFP were analyzed by Bio-Rad MRC1024 laser scanning confocal microscope.

2.7. Transport study in HeLa cells

The transport activity of OCTN2, OCTN2VT, or OCTN2(N91Q) was measured as described previously [9]. In brief, HeLa cells stably expressing YFP (vector alone), OCTN2YFP, OCTN2VTYFP, OCTN2(N91Q)YFP in a 24-well plate were pre-incubated in 0.2 ml of transport buffer (TB) [125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM
CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 25 mM HEPES (pH 7.4)) for 10 min at 37 ºC, and transport reaction was started by addition of 0.2 ml of TB containing 4 nM [³H]carnitine (81 Ci/mmol). After 7 min, the reaction was terminated by aspiration of TB followed by five washes with 1 ml of ice-cold TB. The amount of [³H]carnitine transported into the cells was counted with a liquid-scintillation counter after solubilization of the cells with 0.5 ml of 1% SDS in 0.2 N NaOH. Data were presented as means ± SEM. A pair of means were compared with Student’s t-test. Groups of data were subjected to a two-way analysis of variance (ANOVA), and when a significant F value was encountered, Newman-Keuls’ multiple-range test was used to test for significant differences between treatment means. A probability level of P<0.05 was considered statistically significant.

2.8. Genbank accession number

The accession number for human OCTN2VT is AB291606.
3. Results and discussion

3.1. Isolation and tissue distribution of OCTN2VT

We have recently found a cDNA sequence encoding a variant type of OCTN2 (OCTN2VT) in the database “AssEST”, which is a clustered sequence database containing expressed sequence tags and mRNA sequences in the public domain for human, mouse, rat, and chicken. The putative open reading frame of OCTN2VT was composed of DNA sequence encoding the full-length OCTN2 protein with a DNA-insertion for 24-amino acids (QDSGAYNAMKNRMGKKPALCLPAQ). The insertion was located between Glu-131 and Trp-132 in the first extracellular loop of OCTN2 (Fig. 1A). We searched for the location of the DNA sequence encoding the 24 amino acids in the human genome and found that it was located in the first intron of OCTN2 gene at the chromosome 5 (Fig. 1B). Interestingly, this DNA sequence was interposed between AG and GT (GU in mRNA) dinucleotides, both of which are conserved sequences for splicing of mRNA precursors [14]. Because AG and GU dinucleotides of mRNA precursors are recognized by a spliceosome as a 3’- and 5’-ends of intron, respectively, it is likely that in some case, a spliceosome might recognize the DNA sequence encoding 24 amino acids as an additional exon, leading to an insertion of its DNA sequence between exon 1 and exon 2 to generate OCTN2VT mRNA.

To examine whether mRNA for OCTN2VT is actually expressed in human tissues, we performed PCR using human kidney cDNA library as a template and primers specific for OCTN2VT cDNA. We used two sets of primers: one set corresponds to 5’-end of OCTN2 cDNA and 3’-end of the insert (primers a and b), while the other set corresponds to
5’-end of the insert and 3’-end of OCTN2 cDNA (primers c and d). When these sets of primers were used, PCR products with the expected size were obtained, indicating that mRNA for OCTN2VT is actually expressed. Therefore, we isolated the full-length OCTN2VT cDNA from human kidney cDNA library with PCR. The sequence of the PCR product was completely the same as that of the putative OCTN2VT obtained from the AssEST database. The tissue distribution of OCTN2VT mRNA studied by northern blotting was similar to that of OCTN2 [2] except lower expression in the kidney, indicating that OCTN2VT mRNA is actually expressed in human tissues, and that its gene expression may be regulated in a similar manner to OCTN2.

3.2. OCTN2VT is not an N-glycosylated protein

The first extracellular loop of the OCTN2 is extremely large compared to other extracellular loops (Fig. 1B), and the putative N-glycosylation sites (Asn-57, -64, and -91) are present in this loop [2], suggesting that the first extracellular loop may be critical for the regulation of functional or biochemical properties of OCTN2. Therefore, these properties of OCTN2VT may be altered by the insertion of unique 24 amino acid sequence. First, we investigated the biochemical properties of the OCTN2VT in HeLa cells stably expressing OCTN2\textsuperscript{YFP} or OCTN2VT\textsuperscript{YFP}. Interestingly, the expression level of OCTN2VT, which was analyzed by fluorescent-activated cell sorter (FACS) using fluorescent intensity of YFP as an index, was approximately 10% of the level for OCTN2, despite using the same retroviral expression vector, pMXrmv5 [10] (Fig. 2A and B). The similar result was observed in the immunoblotting (Fig. 2C). Notably, detection of the OCTN2VT protein
required a longer exposure time than that of OCTN2. Because degradation of many proteins is regulated by a ubiquitin-proteasome pathway, we examined whether the OCTN2VT was rapidly degraded by this pathway. As shown in Fig. 2D, MG132, a selective 26S proteasome inhibitor, had no effect on the protein level of the OCTN2VT up to 12 h after its addition, suggesting that the lower level of the OCTN2VT is not due to rapid degradation via the ubiquitin-proteasome pathway.

OCTN2VT was detected as a smaller protein (75 kDa) than OCTN2 (90 kDa) on SDS-PAGE (Fig. 2C), although the molecular weight of OCTN2VT calculated from its amino acid sequence is apparently larger than that of OCTN2 because of the insertion of 24 amino acids (Fig. 1A). Because OCTN2 possesses potential N-glycosylation sites on the first extracellular loop, its larger molecular weight on SDS-PAGE seems to be due to glycosylation of OCTN2. Thus, smaller molecular size of OCTN2VT in comparison with OCTN2 might be due to less or absent N-glycosylation of OCTN2VT.

To verify this point, we compared the apparent molecular weights of OCTN2 and OCTN2VT after treatment of protein extracts with PNGase F, an amidase that cleaves nearly all types of oligosaccharides from glycoproteins. As shown in a right panel of Fig. 2E, the apparent molecular weight of OCTN2 became markedly smaller after treatment with PNGase F (from 90 kDa to 75 kDa), whereas the change in the apparent molecular weight of OCTN2VT was very little. Consequently, after treatment with PNGase F, the apparent molecular weight of OCTN2 became nearly equal to that of OCTN2VT. These results taken together indicate that the different apparent molecular weights of OCTN2 and OCTN2VT reflect abundant glycosylation of OCTN2 and poor glycosylation of OCTN2VT.
Because the insertion of 24 amino acids unique to OCTN2VT was located in the same loop as N-glycosylation sites, this insertion might block N-glycosylation in some way. To get insights into the relationship between N-glycosylation and expression level of OCTN2, we examined a mutant OCTN2 with one of the three potential N-glycosylation sites (Asn-91) being replaced by glutamine (OCTN2(N91Q)): this site was selected because it was the closest to the insertion of 24 amino acids. The expression level of this mutant was found to be reduced to about 50% of a wild type OCTN2 (Fig. 2F and 2G). These results taken together suggest that N-glycosylation may affect the expression level of OCTN2.

3.3. OCTN2VT is retained in the ER

During membrane trafficking for targeting to the plasma membrane, most proteins enter the secretory pathway, i.e. ER and Golgi apparatus, where the proteins are N-glycosylated [15]. Because OCTN2VT was minimally N-glycosylated (Fig. 2D), there is the possibility that OCTN2VT cannot enter the secretory pathway and hence it cannot reach the plasma membrane. To test this possibility, we examined the subcellular localization of OCTN2 and OCTN2VT in Hela cells stably expressing these molecules tagged with YFP. As shown in Fig. 3A, most of OCTN2 proteins were present on the cell surface, indicating that OCTN2 is expressed exclusively on the plasma membrane. In contrast, OCTN2VT proteins were present mainly in the cytoplasm, and its subcellular localization was found to be consistent with that of ER stained with anti-KDEL antibody, which specifically recognizes proteins localized to ER (Fig. 3B), suggesting that OCTN2VT is retained in ER. Since unglycosylated immature proteins are known to
remain in ER to be subjected to proteolysis by the ER quality control system [16], it is likely that OCTN2VT which is lacking N-glycosylation is degraded rapidly because of its localization in ER.

3.4. OCTN2VT is a non-functional transporter for carnitine

To transport carnitine across the plasma membrane, OCTN family proteins must be expressed on the cell surface. However, since OCTN2VT was retained in the ER and not expressed on the cell surface (Fig. 3), it is assumed that OCTN2VT is non-functional as a transporter for carnitine. To test this, we measured the activity of carnitine transport in Hela cells stably expressing OCTN2 and OCTN2VT. In comparison with the cells expressing OCTN2, the cells expressing OCTN2VT showed a very low but statistically significant level of carnitine transport activity: the uptake into the cells expressing OCTN2VT and vector alone were 658 ± 9 and 603 ± 6 dpm/3x10^5 cells, respectively (n=3, P<0.05) (Fig. 4A). The difference of these two values is a net uptake via OCTN2VT, which is about 0.20% of the uptake via OCTN2. After normalization of expression levels of OCTN2VT by MFI, the uptake by OCTN2VT is still far lower than the uptake by OCTN2. Considering that the MFI value obtained by FACS Calibur reflects expression levels of OCTN2VT in whole areas of individual cells but not on cell surface alone, and also that OCTN2VT is localized mainly in cytosol, the transport activity of each OCTN2VT molecule is estimated to be far higher than this estimated value. Since it is at present difficult to estimate the precise proportion of OCTN2VT expressed on the cell surface to total OCTN2VT expressed in the whole cell, the actual transport activity of each
OCTN2VT molecule is unknown. Taken together, these results indicate that a minute amount of OCTN2VT with a carnitine transport activity is expressed, although it is unknown whether the activity of each OCTN2VT molecule is normal or reduced in comparison with that of wild type OCTN2.

To examine the relationship among N-glycosylation, expression level, and transport activity of OCTN2, we also analyzed the carnitine transport activity of OCTN2(N91Q): its expression level was about half of OCTN2 (Fig. 2F and 2G) and most of it was present on the cell surface (Fig. 4A, inset). The carnitine uptake into the cells expressing OCTN2(N91Q) was about half of OCTN2 (Fig. 4A). However, after normalization of its expression level by MFI, the transport activity was found to be not significantly different from the cells expressing OCTN2 (OCTN2(N91Q), 33.6 ± 2.1 x 10^3 dpm/3x10^5 cells; OCTN2, 26.7 ± 1.3 x 10^3 dpm/3x10^5 cells: P>0.05). These results imply that the reduction of glycosylation affects the expression level but not the transport activity of OCTN2.

A variety of mutations for OCTN2 have been reported in the systemic carnitine deficiency (SCD) of humans [5], [17], [18], [19], and [20]. The Phe-17 to Leu mutation of OCTN2 identified in the SCD patient showed a reduction of $V_{max}$ with no effect on $K_m$ for carnitine transport due to its failure to be expressed on the cell surface [21]. Furthermore, Amat di San Filippo et al have studied eight families with SCD and found several point mutations at sites other than glycosylation sites, which caused significant retention of the mutant OCTN2 in the cytoplasm [22]. Our results and these reports suggest that the subcellular localization of the OCTN family proteins is a critical factor for the pathogenesis
of SCD.

Although it is not clear whether OCTN2VT transports carnitine if expressed on the plasma membrane, its actual function might transport organic cations across the ER membrane. It would provide new insights into the physiological functions of the OCTN family proteins to identify the substrate molecules transported by OCTN2VT.

3.5. Concluding remarks

Although the OCTN family proteins have been thought to be well-conserved with respect to the biochemical and functional characteristics, the OCTN2VT protein isolated in this study showed unique characteristics, which are totally distinct from the OCTN2 and other OCTN family proteins. Our results demonstrate that OCTN2VT did not have the typical characteristics of OCTN family proteins, such as an N-glycosylation and cell surface expression, due to the insertion of 24 amino acids in the first extracellular loop of OCTN2. Furthermore, the Hela cells expressing OCTN2VT proteins could not take up carnitine due to the retention of OCTN2VT proteins in the ER. The insertion of 24 amino acids may be made by the alternative splicing because the DNA sequence encoding the 24 amino acids was interposed between the conserved splice sites. Further investigation into the physiological functions of OCTN2VT in the ER may reveal the novel transport activity of OCTN family proteins across the ER membrane as well as the plasma membrane.
Acknowledgements

This work was supported by Grants-in-Aid and Special Coordination Funds for Promoting Science and Technology, from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from the Smoking Research Foundation, Japan.
References


Legends

Fig. 1. Identification of a splice variant of OCTN2. (A) Alignment of OCTN2 and OCTN2VT proteins. The insertion of 24 amino acids in the OCTN2VT is shown in red in both amino acid sequence and the predicted secondary structure. The protein encoded by exon 2 is shown in blue. The three putative N-glycosylation sites are indicated by asterisks. The putative transmembrane domains (TM1, TM2, and TM3) are shown in boxes. (B) OCTN2VT generated from splicing of OCTN2 gene is illustrated. The genome sequence corresponding to the illustration is shown below. The DNA sequences of the spliced region and exon 2 are shown in red and blue, respectively. The GT-AG intron termini are shown in orange box. (C) Expression of OCTN2VT mRNA in the human kidney. Primers used for RT-PCR are shown by arrows. Primers b and c are specific for OCTN2VT cDNA. (D) Tissue distribution of OCTN2VT mRNA detected by northern blot analysis.

Fig. 2. Comparison of biochemical properties of OCTN2, OCTN2VT, and OCTN2(N91Q). (A) and (B) Expression levels of OCTN2 and OCTN2VT in HeLa cells following transfection of cDNA. OCTN2 and OCTN2VT tagged with YFP (designated OCTN2\textsubscript{YFP} and OCTN2VT\textsubscript{YFP}) were expressed in Hela cells using retroviral vectors: in control cells, no transfection (designated HeLa). Fluorescent intensity of YFP in each cell was analyzed by FACS, and represented as a plot of fluorescent intensity in each cell (ordinate) against FSC (abscissa) (A) or mean fluorescent intensity (MFI) (B). (C) Immunoblot analysis of membrane fraction prepared from Hela cells expressing OCTN2\textsubscript{YFP} (WT) or OCTN2VT\textsubscript{YFP}.
(VT) by anti-GFP antibody. Left and right panels represent the results with a short or long exposure time, respectively. (D) Immunoblot analysis for changes of expression level of OCTN2\textsubscript{VT}\textsuperscript{YFP} in Hela cells following 4-h, 8-h, and 12-h treatment with 10 mM MG132, a proteasome inhibitor. (E) Immunoblot analysis as in panel C. Unlike panel C, membrane fractions prepared from Hela cells expressing OCTN2\textsuperscript{YFP} (WT) and OCTN2\textsubscript{VT}\textsuperscript{YFP} (VT) were incubated with PNGase F for 1 h at 37 °C and subsequently subjected to SDS-PAGE. (F) and (G) Expression levels of OCTN2\textsuperscript{YFP} and OCTN2(N91Q)\textsuperscript{YFP} in Hela cells following transfection of cDNA were analyzed by FACS.

**Fig. 3.** Subcellular localization of OCTN2\textsuperscript{YFP} (A) and OCTN2\textsubscript{VT}\textsuperscript{YFP} (B). For staining of endoplasmic reticulum, Hela cells expressing OCTN2\textsuperscript{YFP} and OCTN2\textsubscript{VT}\textsuperscript{YFP} were first incubated with anti-KDEL antibody (right panels) after fixation and permeabilization of the cells, and subsequently, they were incubated with Alexa Fluor 546-conjugated anti-mouse IgG for anti-KDEL antibody. After treatments, fluorescent images of the cells derived from YFP (left panels) and Alexa Fluor 546 (middle panels) were obtained using a Bio-Rad MRC1024 laser scanning confocal microscopy. Right panels are merged images.

**Fig. 4.** Carnitine transport activity of OCTN2\textsuperscript{YFP}, OCTN2\textsubscript{VT}\textsuperscript{YFP}, and OCTN2(N91Q)\textsuperscript{YFP}. (A) Hela cells stably transfected with expression vectors containing cDNAs for either YFP (vector), OCTN2\textsuperscript{YFP}, OCTN2\textsubscript{VT}\textsuperscript{YFP}, and OCTN2(N91Q)\textsuperscript{YFP} were incubated with a saturating concentration (4 nM) of \textsuperscript{3}H\textsubscript{carnitine} (81 Ci/mmol) for 7 min at 37 °C. \textsuperscript{3}H\textsubscript{carnitine} uptake into these cells were shown as mean ± SEM (n=3). Inset: The
subcellular distribution of OCTN2(N91Q)YFP. (B) Summary of the present study.
Fig. 1_Maekawa et al.
Fig. 2_Maekawa et al.
Fig. 3_Maekawa et al.
**A**

Carnitine uptake ($\times 10^3$ dpm/$3 \times 10^5$ cells/7 min)

<table>
<thead>
<tr>
<th></th>
<th>Vector</th>
<th>WT</th>
<th>VT</th>
<th>N91Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\times 10^3$ dpm</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

**B**

Fig. 4 Maekawa et al.