Molecular and Functional Identification of Sodium Ion-dependent, High Affinity Human Carnitine Transporter OCTN2*

Ikumi Tamaï, Rikiya Ohashi, Jun-ichi Nezu, Hikaru Yabuuchi, Asuka Oku, Miyuki Shimane, Yoshimichi Sai, and Akira Tsuji*†

From the ‡Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan and the §Chugai Research Institute for Molecular Medicine Inc., Ibaraki 300-4101, Japan

EXPERIMENTAL PROCEDURES

Materials—t-[(Methyl-3H)carnitine hydrochloride (85 Ci/mmol) and [14C]guanidine (56 mCi/mmol), [1-14C]-tetraethylammonium bromide (2.4 mCi/mmol), and [α-32P]dCTP were purchased from Amersham Pharmacia Biotech (Rockinghamshire, UK), Moravek Biochemicals Inc. (Brea, CA), and New England Nuclear (Boston, MA), respectively. pcDNA3 was obtained from Invitrogen (San Diego, CA). Multiple tissue Northern blots were purchased from CLONTECH. All other enzymes and reagents were obtained from Takara (Otsu, Japan), Toyobo (Osaka, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Sigma Chemical Co. (St. Louis, MO). HEK293 cells were obtained from Japa
genese Cancer Research Resources Bank (Tokyo, Japan).

Cloning of OCTN2 cDNA and Northern Blot Analysis—A data base search for matches to the cDNA sequence of the OCTN1 gene revealed several genomic cosmids clones (GenBank accession numbers L43407, L43408, L46907, L81773, and L43409), derived from human chromosome 5q, that contain sequences highly homologous to OCTN1. Because these genomic sequences do not cover the entire open reading frame for this new gene, which we designated OCTN2 on the basis of its high similarity to OCTN1, we initiated cDNA cloning. From the genomic

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1 The abbreviation used is: TEA, tetraethylammonium.

‡ To whom correspondence should be addressed. Fax: 81-76-234-4477; E-mail: tsuji@kenroku.ipc.kanazawa-u.ac.jp.

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sequence, two primers (631RT S4 5′-AGCTGCATGAAGAGAAGGACACTG-3′ and 631RT A1 5′-AGCTGCATGAAGAGAAGGACACTG-3′) were prepared and used in reverse transcription-polymerase chain reaction of human kidney-derived cDNA. This afforded a 900-base pair 3′ cDNA fragment of OCTN2. Screening of a human kidney cDNA library with this fragment as the probe yielded overlapping, longer clones that provided additional sequences. A primer (631R S6 5′-AGCATCTTGTTGGCTTCCTACTTTCA-3′ and 631R T4 5′-AGCTGCATGAAGAGAAGGACACTG-3′) was designed from the new sequence was used to amplify the 3′ portion of OCTN2 by 3′ rapid amplification of cDNA ends using human kidney Marathon-ReadyTM cDNA (CLONTECH).

Finally, the full coding sequence of OCTN2 was obtained by assembling these sequences. To assess OCTN2 expression in human tissues, an OCTN2 cDNA fragment, amplified with the 631R T4 and 631RT A1 primers, was labeled with [α-32P]dCTP and subjected to Northern blotting with poly(A)+ RNA from a wide range of normal human tissues and cancer cell lines (CLONTECH). Hybridization was carried out in ExpressHyb hybridization solution (CLONTECH) at 68 °C for 1 h. Membranes were then washed in 2× SSC containing 0.1% SDS at room temperature for 60 min and finally in 0.1× SSC, 0.1% SDS at 50 °C for 20 min twice.

Transport Study in HEK293 Cells—The full-length OCTN2 cDNA was subcloned into the BamHI sites of the expression vector pcDNA3, and the construct, pcDNA3/OCTN2 was used to transfect HEK293 cells by means of the calcium phosphate precipitation method as described previously (17). The cells were cultivated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc., Tokyo, Japan). Cellular protein content was determined according to the method of Bradford (19) using a Bio-Rad protein assay kit. When radioactivity was quantitated in a liquid scintillation counter (Aloka, Tokyo, Japan), 100 units/ml penicillin, and 100 μg/ml streptomycin in tissue culture dishes in a humidified incubator at 37 °C under 5% CO2 for 24 h and then transfected with pcDNA3 plasmid carrying full-length OCTN2 cDNA or with the pcDNA3 plasmid vector alone. At 48 h after transfection, the cells were harvested by scraping with a rubber policeman and suspended in the medium for transport study, which consisted of 125 mM NaCl, 4.8 mM KCl, 5.6 mM d-glucose, 1.2 mM CaCl2, 1.2 mM MgCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 25 mM HEPES (pH 7.4). The cell suspension and transport medium containing a radiolabeled test compound were preincubated separately for 20 min and then mixed to initiate uptake. At appropriate times, 200-μl aliquots of the mixture were withdrawn, and the cells were separated from the transport medium by centrifugation in a microtube containing a silicon oil and liquid paraffin mixture with a density of 1.03. The resultant cell pellets were solubilized in 3 N KOH and then neutralized with HCl, and the associated radioactivity was quantitated in a liquid scintillation counter (Aloka, Tokyo, Japan).

The criterion of significance was taken to be p < 0.05. The uptake rate (v) was fitted to the following equation by means of nonlinear least squares regression analysis using WinNonlin (Scientific Consulting Inc., Cary, NC). v = Vmax × s/(Ks + s), where v and s are the uptake rate and concentration of carnitine, respectively, and Ks and Vmax are the half-saturation concentration (Michaelis constant) and maximum transport rate, respectively. All data were expressed as the mean ± S.E., and statistical analysis was performed by use of Student’s t test. The criterion of significance was taken to be p < 0.05.

RESULTS

Amino Acid Sequence and Tissue Distribution of Human OCTN2—The full-length OCTN2 cDNA appeared to encode a polypeptide of 557 amino acids and have 75.8% similarity with human OCTN1 (17) (Fig. 1A). Human OCTN2 is predicted to have twelve putative membrane-spanning domains by hydropathy analysis according to TopPred 2 (20) as well as three N-glycosylation sites and six protein kinase C phosphorylation sites. The presence of twelve membrane spanning domains agrees with that of previously known membrane transporters (21). Like human OCTN1, OCTN2 has a unique sugar transport protein signature (17). Comparison of the amino acid sequence with those of other organic ion transporters revealed that human OCTN2 has similarity with rat OCT1 (32.5%) (22), rat OCT2 (33.6%) (23), human OCT1 (33.1%) (24), human OCT2 (33.1%) (25), and rat OAT1 (28.4%) (26). No significant similarity was observed with human oaat (27) or monooamine neurotransmitter transporters such as serotonin transporter (28) or monoamine transporter (29). These data indicate that OCTN2 may be a member of the organic cation transporter family.

Fig. 2 shows the tissue distribution of human OCTN2 examined by Northern blotting analysis. In fetal tissues, OCTN2 was expressed strongly in kidney and weakly in liver, lung, and brain. In adult, it was expressed strongly in kidney, skeletal muscle, placenta, heart, prostate, and thyroid and weakly in pancreas, liver, lung, brain, small intestine, uterus, thymus, adrenal gland, trachea, spinal cord, and several other tissues. Interestingly, specific bands corresponding to human OCTN2 were also detected in human tumor-derived cell lines, including melanoma G361, lung carcinoma A549, colorectal carcinoma SW480, chronic myelogenous leukemia K562, and carcinoma of cervix HeLa S3. The distribution in tumor cell lines is very similar to that of human OCTN1. On the other hand, the distributions of OCTN2 in normal fetal and adult tissues are different from those of OCTN1, especially as regards the significant expression of OCTN2 in fetal kidney and in skeletal muscle, liver, placenta, heart, prostate, and thyroid in adults, although OCTN1 and OCTN2 both exhibit broad tissue distribution.

Functional Analysis of OCTN2 Expressed in HEK293 Cells—Because human OCTN1 transported the organic cation TEA in a pH-dependent manner when expressed in HEK293 cells in our previous study (17), we expressed human OCTN2 in the same cells and measured the uptake of cationic compounds for comparison with that of OCTN1. Although TEA is a good substrate of OCTN1 (17), no significant increase of [14C]TEA uptake was observed in human OCTN2-transfected cells (298 ± 60 pmol/mg protein/3 min in OCTN2-transfected HEK293 cells...
and 263 ± 12 in nontransfected cells at the TEA concentration of 60 μM and at 3 min). Because guanidine was suggested to be transported by a different transporter than that for TEA across the apical membrane of renal tubular epithelial cells (30), we examined the uptake of [14C]guanidine in human OCTN1- or OCTN2-expressing cells. However, neither OCTN1 (106 ± 5.6 pmol/mg protein/3 min at 10 μM guanidine) nor OCTN2 (95.1 ± 11.8 pmol/mg protein/3 min at 10 μM guanidine) exhibited increased guanidine transport activity compared with nontransfected cells (98.4 ± 5.9 pmol/mg protein/3 min at 10 μM guanidine). Zwitterionic carnitine is reabsorbed in the kidney via an active transport mechanism, although the molecular identity of the transporter remains to be established. Accordingly, we examined the transport of carnitine in the present study. Although OCTN1 showed slight but significant uptake of L-[3H]carnitine (2.5-fold increased uptake compared with nontransfected cells), a very large uptake of L-[3H]carnitine was seen with the human OCTN2-expressing HEK293 cells, as described below.

Fig. 3A shows the time course of the uptake of L-[3H]carnitine by HEK293 cells transfected with OCTN2 or with the expression vector pcDNA3 alone, in the presence or absence of sodium ions. Uptake of L-[3H]carnitine was significantly increased by OCTN2 transfection both in the presence and absence of sodium ions in the transport medium. The uptake of L-[3H]carnitine was particularly high in OCTN2-transfected cells in the presence of sodium ions, and it appears that OCTN2 is a sodium ion-dependent carnitine transporter. Because the cells transfected with expression vector alone showed a slight but significant increase of L-[3H]carnitine uptake in the presence of sodium ions compared with that in the absence of sodium ions, HEK293 cells themselves seem to have a weak activity of sodium ion-dependent carnitine transport. This is not surprising considering that HEK293 cells were originally derived from human embryonic kidney. Because sodium ion-dependent and OCTN2-mediated uptake of carnitine increased linearly up to 5 min, initial uptake rate of carnitine was determined at 3 min to characterize the transporter in all subsequent studies.

To confirm the driving force for OCTN2-mediated carnitine transport, the effect of sodium ion replacement with various cations on carnitine transport was examined. When sodium was replaced with N-methylglucamine, potassium, or choline, initial uptake of L-[3H]carnitine was almost abolished, whereas substitution with lithium resulted in retention of a weak uptake activity (Fig. 3B). When the temperature of the transport assay was decreased to 4 °C, the initial uptake was decreased to 3.15 ± 0.50% of that at 37 °C, demonstrating a significant temperature dependence of transport with an estimated activation energy of 17.9 kcal/mol.

The concentration dependence of L-carnitine transport was examined to estimate the half-saturation concentration of...
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CONCENTRATION DEPENDENCE AND SUBSTRATE SPECIFICITY OF CARNITINE TRANSPORT BY OCTN2 EXPRESSED IN HEK293 CELLS. A, uptake of carnitine at various concentrations was measured at pH 7.4 and 37 °C for 3 min. B, Eadie-Hofstee plot of the concentration dependence of carnitine uptake by OCTN2 expressed in HEK293 cells. C, inhibition by test compounds at various concentrations (in μM) and substrate specificity of OCTN2-mediated carnitine transport was examined in terms of the inhibitory effect on the initial uptake of L-[3H]carnitine. As is clearly shown in Fig. 4C, structurally analogous compounds, D-carnitine, acetyl-D,L-carnitine, and γ-butyrobetaine reduced L-[3H]carnitine uptake at 5 and 50 μM. Because L-carnitine demonstrated more potent inhibition than the D-isomer at low concentration (5 μM) OCTN2 seems to have stereospecificity. Glycinobetaine less effectively reduced the uptake of L-[3H]carnitine, and others examined, including γ-aminobutyric acid, choline, β-hydroxybutyrate, lysine, and taurine, were not inhibitory. Accordingly, the structural requirement of OCTN2-mediated carnitine transport is rather strict.

DISCUSSION

Carnitine is normally maintained at a steady level in the blood, suggesting its physiological importance (1, 2). Although carnitine is biosynthesized in liver and brain (31), a significant amount of carnitine is also obtained from the diet via carrier-mediated transport across the intestinal epithelial cell membranes (32, 33) and is retained in the body through reabsorption in the kidney via active transport across the renal tubular epithelial cell membrane (1, 7, 14, 15). Furthermore, many studies have demonstrated that the tissues that extensively accumulate carnitine, such as skeletal muscle, heart, liver, and epididymis, take up and/or release carnitine via specialized transport mechanisms to maintain the steady-state tissue concentration (1). Although many of these membrane physiological studies suggested the participation of multiple sodium ion-dependent transporters with high and low affinities in carnitine movement across tissue plasma membranes (1, 13, 15), no such transporter has yet been identified at the molecular level. Accordingly, to achieve a better understanding of the biological and physiological roles of carnitine, as well as carnitine-related pathological states, it is essential to identify the carnitine transporter(s). We have previously cloned the human organic cation transporter OCTN1, which may participate at least partially in proton/organic cation antiport at the renal apical membranes, and characterized it by measuring the uptake of the typical organic cation TEA by OCTN1-transfected HEK293 cells (17). In the present study, we succeeded in obtaining cDNA of a second member of the human OCTN family, OCTN2, which has a high similarity to OCTN1, and found that OCTN2 has many of the characteristics of a high affinity, sodium ion-dependent carnitine transporter.

The idea that human OCTN2 is a sodium ion-dependent carnitine transporter is supported by the specific tissue distribution and the result of functional expression in HEK293 cells. Most adult tissues that highly express OCTN2, including skeletal muscle, kidney, placenta, and heart, have been reported to take up carnitine via a sodium ion-dependent, carrier-mediated transport mechanism (1, 15, 16, 34–36). As regards fetal tissues, we previously found that mouse embryo fibroblasts take up carnitine in a sodium ion-dependent manner with a half-saturation concentration of 5.5 μM, although the distribution of carnitine transport activity in fetal tissues was not established (13). Tissues that have apparently low affinity carnitine transporters, such as liver, brain, and intestine, with apparent half-saturation concentrations between 0.2 mM and 10 mM (1, 33, 37, 38), showed low expression of OCTN2. The distribution of OCTN2 is different from that of OCTN1, which has 75.8% sequence similarity with OCTN2 and coincides well with the functional distribution of sodium ion-dependent, high affinity carnitine transport activity as discussed above. Furthermore, OCTN2 hardly transported TEA, a good substrate of OCTN1, or guanidine, a substrate of the second renal organic cation transporter, which is distinct from that for TEA (30). OCTN2 seems likely to have some physiological role other than the renal excretion of organic cations.

When OCTN2 was expressed in HEK293 cells, a high uptake of L-[3H]carnitine was observed in the presence of sodium ions (Fig. 3, A and B). Because the cellular volume of HEK293 cells obtained from the accumulation of 3H2O is 6.3 μl/mg protein (17), L-[3H]carnitine apparently accumulated to the extent of about 320-fold at the steady-state in the cells by utilizing an inside-directed sodium ion gradient as the driving force. Lithium ions partially retained uphill transport (Fig. 3B) in a manner comparable with the carnitine transport obtained using rat renal brush border membrane vesicles (15) and human placental choriocarcinoma cells (16), which suggests that lithium ions are partially accepted as the cation for cotransport with carnitine. When sodium was replaced with choline, carnitine uptake was specifically eliminated (Fig. 3B). Although 500
μM choline was not inhibitory (Fig. 4B), choline may have a low affinity to compete with carnitine binding to OCTN2.

The half-saturation concentration of L-carnitine uptake by OCTN2 was estimated to be 4.34 μM, which is very similar to the values observed for high affinity carnitine transport in membrane physiological studies in kidney, skeletal muscle, heart, placenta, and cultured fibroblasts (1, 2, 5, 6, 15, 16, 34–36), tissues that exhibited high expression of OCTN2 in heart, placenta, and cultured fibroblasts (1, 2, 5, 6, 15, 16, 34–36), tissues that exhibited high expression of OCTN2 in Northern blot analysis (Fig. 2). Carnitine transport in these tissues was reported to be significantly inhibited by the D-isomer of carnitine, acetylcarnitine, and γ-butyrobetaine in a stereospecific manner (6, 15, 16, 35, 36). Furthermore, glycine-betaine and choline were low affinity inhibitors and γ-aminobutyric acid was not inhibitory (14–16, 36). These previously reported substrate specificity characteristics exactly coincide with the properties of OCTN2. We conclude that OCTN2 is a high affinity, sodium ion-dependent carnitine transporter expressed in several tissues, including kidney, skeletal muscle, heart, and placenta.

The amino acid sequence of human OCTN2 is very similar to that of human OCTN1, although OCTN1 exhibited only a low carnitine transport activity and had the functional characteristics of a proton/organic cation antiporter (17). Furthermore, TEA, a good substrate for OCTN1, was not transported well by OCTN2. Such significant differences of substrate specificity for substrates and cotransported ions on the transporter proteins is determined in a very limited region and that OCTN proteins have both common structures as membrane transporters and distinct small regions for the recognition of the substrate and cotransported ions. It will be interesting to identify the essential amino acid sequence of the functional binding sites by constructing chimeric proteins of OCTN1 and OCTN2. The strong expression of OCTN2 in human-derived tumor cells is similar to that of OCTN1 (14). This may reflect up-regulation of expression of the gene in malignancy to meet a higher requirement for l-carnitine.

In conclusion, human OCTN2 was cloned as a new member of the family of organic cation transporters. Studies of its tissue distribution and its functional expression in HEK293 cells indicated that OCTN2 is a physiologically important, high affinity carnitine transporter that shows significant sodium ion dependence. The identification of this carnitine transporter should contribute to a better understanding of the physiological and biochemical functions of carnitine, as well as to the development of measures to treat primary carnitine deficiency. In addition, isolation of the counterpart of OCTN2 in mice and comparison with juvenile visceral steatosis mice, which show abnormal carnitine metabolism and related diseases, may help to identify the principal causes of carnitine deficiency syndromes (7, 9–12).

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