Mutations in Novel Organic Cation Transporter (OCTN2), an Organic Cation/Carnitine Transporter, with Differential Effects on the Organic Cation Transport Function and the Carnitine Transport Function*

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Novel organic cation transporter (OCTN2) is an organic cation/carnitine transporter, and two missense mutations, L352R and P478L, in OCTN2 have been identified as the cause for primary carnitine deficiency. In the present study, we assessed the influence of these two mutations on the carnitine transport function and the organic cation transport function of OCTN2. The L352R mutation resulted in a complete loss of both transport functions. In contrast, the P478L mutation resulted in a complete loss of only the carnitine transport function but significantly stimulated the organic cation transport function. Studies with human OCTN2/rat OCTN2 chimeric transporters indicated that the carnitine transport site and the organic cation transport site were not identical. Because carnitine transport is Na+-dependent whereas organic cation transport is Na+-independent, we investigated the possibility that the P478L mutation affected Na+ binding. The Na+ activation kinetics were found to be similar for the P478L mutant and wild type OCTN2. We then mutated nine different tyrosine residues located in or near transmembrane domains and assessed the transport function of these mutants. One of these mutations, Y211F, was found to have differential influence on the two transport activities of OCTN2 as did the P478L mutation. However, the Na+ activation kinetics were not affected. These findings are of clinical relevance to patients with primary carnitine deficiency because whereas each and every mutation in these patients is expected to result in the loss of the carnitine transport function, all of these mutations may not interfere with the organic cation transport function.

OCTN2 is a member of the organic cation transporter family (1). It was first cloned from human placenta and was shown to transport organic cations (2). Subsequently, this transporter was also shown to mediate the Na+-dependent uptake of carnitine (3, 4). Interestingly, the transport of carnitine via OCTN2 is a Na+-coupled process whereas the transport of organic cations via OCTN2 is Na+-independent. To our knowledge, this is a unique finding among the Na+-coupled organic solute transporters cloned thus far. No other transporter has been shown to transport some substrates in a Na+-dependent manner and other substrates in a Na+-independent manner. Even within the family of the organic cation transporters to which OCTN2 belongs, no other member of the family exhibits this intriguing characteristic. Carnitine plays an obligatory role in fatty acid oxidation (5), and Na+-coupled transport of carnitine via OCTN2 is essential for the maintenance of high concentrations of carnitine inside the cells (6). OCTN2 is widely expressed in human tissues including the heart, skeletal muscle, kidney, intestine, and placenta (2–4), and the physiological relevance of the carnitine transport function of OCTN2 in these tissues is readily recognizable (7–11). The finding that OCTN2 also mediates the cellular uptake of organic cations indicates that the transporter may have hitherto unrecognized functions of significant pharmacological relevance.

Genetic defects in OCTN2 are the cause of primary carnitine deficiency (12–16). The clinical symptoms of this disease include cardiomyopathy, progressive muscle weakness, non-ketotic hypoglycemia, and hyperammonemia. The severity of the clinical outcome of defects in OCTN2 is further supported by a mouse model, known as the juvenile visceral steatosis (jvs) mouse, that is defective in OCTN2 and develops microvesicular fatty infiltration of the viscera and marked cardiomyopathy (16, 17). Most of the mutations in OCTN2 identified in humans with primary carnitine deficiency result in truncated proteins and loss of carnitine transport function (12–15). To our knowledge, only one missense mutation, P478L, has been identified in human patients (13). The mutation in jvs mouse has recently been reported (16), and it is also a missense mutation (L352R). Both of these mutations are associated with the loss of carnitine transport function (4, 13), which explains the clinical consequences of these mutations. The present investigation was undertaken to investigate the influence of these two missense mutations on the organic cation transport function. These studies have led to a very interesting observation that these mutations have differential effects on the carnitine transport function versus the organic cation transport function. Whereas the L352R mutation causes a complete loss of both transport functions, the P478L mutation interferes only with the carnitine transport function. These interesting observations formed the basis for additional studies with cross-species chimeric OCTN2 transporters and several new OCTN2 mutants. These studies demonstrate that the organic cation transport site and the carnitine transport site overlap but are not identical.

EXPERIMENTAL PROCEDURES

Materials—[L-3H]Carnitine (specific radioactivity, 65 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA), and [ethyl-1-14C]tetraethylammonium (TEA) bromide (specific radioactivity, 55
mCi/mmol was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Cell culture media and Lipofectin were from Life Technologies, Inc. Restriction enzymes were from New England Biolabs (Beverly, MA).

Site-directed Mutagenesis—The QuickChange site-directed mutagenesis kit (Stratagene) was used to generate the human OCTN2 and rat OCTN2 mutants according to the manufacturer’s protocol, and the details of the procedure have been described previously (4). The entire coding region of the mutant cDNAs was sequenced using an automated Perkin-Elmer Applied Biosystems 377 Prism DNA sequencer to confirm the presence of the introduced mutations and the absence of any unwanted mutations arising from polymerase chain reactions. A total of 13 mutants were generated: M352R and P478L of human OCTN2, L352R and P478L of rat OCTN2, and nine tyrosine mutants of rat OCTN2 (Y211F, Y239F, Y251F, Y358F, Y426F, Y479F, Y482F, Y486F, and Y492F).

Construction of Cross-species OCTN2 Chimeras—Cross-species chimeras were constructed between human OCTN2 and rat OCTN2 by swapping specific restriction fragments between the two cDNAs. This was made possible because of the presence of several homologous regions at the nucleotide level in the coding region between human OCTN2 and rat OCTN2. A total of six chimeras were constructed. Chimeras 1 and 2 were constructed with the splice junction at the amino acid position 448/450 by using the BsrGI restriction site. Chimeras 3 and 4 were constructed with the splice junction at the amino acid position 340 by using the NsiI restriction site. Chimeras 5 and 6 were constructed with the splice junction at the amino acid position 122/123 by using the AvaII restriction site. The splice junctions of all of the chimeras were in-frame and did not alter the reading frame. Each of the chimeras was sequenced at the splice site for confirmation of the switching of the cross-species restriction fragments.

Functional Expression of OCTN2 cDNAs—The wild type, mutant, or chimeric OCTN2 cDNAs were oriented in the pSPORT vector in such a way that their sense transcription was under the control of the T7 promoter. The cDNAs were heterologously expressed in HeLa cells by using the vaccinia virus expression technique as described previously (2, 4). Transport measurements were made 12 h after transfection. Transport of [14C]TEA or [14C]carnitine was measured at room temperature with a 30-min incubation. The composition of the transport buffer was 140 mM NaCl (Na+-containing buffer) or 140 mM N-methyl-D-glucamine chloride (Na+-free buffer), 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, and 5 mM glucose. The Na+-containing medium was buffered with 25 mM Hepes/Tris, pH 7.5. The Na+-free medium was buffered with either 25 mM Hepes/Tris, pH 7.5 or 25 mM Hepes/Tris, pH 8.5. The transport medium containing varying concentrations of Na+ was prepared by appropriately mixing the Na+-containing buffer and the Na+-free buffer. Transport was terminated by aspiration of the medium followed by two quick washes with 2 ml of ice-cold transport buffer. The cells were then solubilized with 0.5 ml of 1% SDS in 0.2 M NaOH and transferred to vials for quantitation of radioactivity associated with the cells. HeLa cells transfected with vector alone under similar conditions served as control.

RESULTS AND DISCUSSION

Influence of L352R/M352R and P478L Mutations on the Carnitine Transport Function and Organic Cation Transport Function—The L352R mutation was identified in the jvs mouse, an animal model for primary carnitine deficiency (16). A comparison of amino acid sequences of wild type OCTN2s shows that the amino acid at position 352 is leucine in rat OCTN2 as it is in mouse OCTN2, but the corresponding amino acid in human OCTN2 is methionine. The P478L mutation was identified in a patient with primary carnitine deficiency (13). The proline residue at position 478 is conserved in wild type OCTN2s of all three species (mouse, rat, and human). To assess the influence of these mutations on transport function, we introduced these mutations in rat and human OCTN2s by site-directed mutagenesis and expressed the mutants (M352R of human OCTN2, L352R of rat OCTN2, and P478L of rat and human OCTN2s) and the corresponding wild type OCTN2s in HeLa cells (Fig. 1). The carnitine transport function was assessed by measuring the transport of 20 nM carnitine at pH 7.5 in the presence of Na+, and the organic cation transport function was assessed by measuring the transport of 20 μM TEA at pH 8.5 in the absence of Na+. These experimental conditions were chosen for optimal transport of the two substrates. The M352R mutation in human OCTN2 and the L352R mutation in rat OCTN2 completely abolished the transport of carnitine. Similarly, the P478L mutation in rat and human OCTN2s also abolished the carnitine transport function by >95%. However, the influence of these mutations on the organic cation transport function was different. The M352R mutation in human OCTN2 and the L352R mutation in rat OCTN2 caused a loss of >90% of TEA transport whereas the P478L mutation did not interfere with TEA transport. In fact, TEA transport was significantly higher in the case of the mutant OCTN2 than the wild type OCTN2. The increase in TEA transport as a consequence of the P478L mutation was 3.7-fold for human OCTN2 and 1.3-fold for rat OCTN2.

Influence of M352R/L352R and P478L mutations on carnitine transport and TEA transport. The wild type and mutant OCTN2 cDNAs (human and rat) were expressed heterologously in HeLa cells. Transport of carnitine (20 nM) was measured at pH 7.5 in the presence of Na+. Transport of TEA (20 μM) was measured at pH 8.5 in the absence of Na+. Transport measured in cells transfected with vector alone was subtracted from transport in cDNA-transfected cells to calculate cDNA-specific transport. WT, wild type.
Effects of Mutations in OCTN2 on Transport Function

Influence of P478L Mutation on Na⁺ Activation Kinetics of Carnitine Transport—Because carnitine transport via OCTN2 is Na⁺-dependent whereas TEA transport via OCTN2 is Na⁺-independent, a possible explanation for the differential effect of the P478L mutation on the transport of these two substrates is that the mutation lies in the Na⁺-binding site and hence interferes with carnitine transport without affecting TEA transport. To test this possibility, we analyzed the Na⁺ activation kinetics of carnitine transport for wild type rat OCTN2 and the P478L mutant of rat OCTN2. Even though the rat mutant possessed only about 3% of carnitine transport activity of the wild type rat OCTN2 and the Y211F mutant of rat OCTN2. When the carnitine transport activity was affected markedly by the mutation of a potential Na⁺-binding site, we mutated each of these nine tyrosyl residues and assessed the influence of these mutations on carnitine transport and TEA transport. These experiments identified two tyrosine mutations, Y358F and Y482F, that abolished the carnitine transport function as well as the TEA transport function (Table I). One mutant, Y211F, exhibited the characteristics expected of a mutation in the Na⁺-binding site. This mutation caused a 90% loss of carnitine transport but did not interfere with TEA transport. The remaining tyrosine mutations did not affect OCTN2 function. We then analyzed the Na⁺ activation kinetics of the residual carnitine transport of the Y211F mutant. Contrary to our expectations, the Na⁺ activation kinetics of the mutant were similar to that of wild type OCTN2 even though the carnitine transport activity was reduced markedly by the mutation (Fig. 3). The $K_{0.5}$ for Na⁺ activation was $8.9 \pm 0.4$ mM. Thus, the P478L mutant and the Y211F mutant exhibited similar features. Both mutations affected only carnitine transport without interfering with TEA transport and Na⁺ activation kinetics. The Y358F and Y482F mutants, on the other hand, were similar to the

### Table I

| cDNA      | TEA transport | Carnitine transport |
|-----------|---------------|---------------------|
| Wild type | 189.2 ± 2.9   | 1.63 ± 0.10         |
| Y211F    | 216.3 ± 41.1  | 0.16 ± 0.00         |
| Y239F    | 169.9 ± 5.7   | 1.33 ± 0.23         |
| Y251F    | 217.7 ± 10.9  | 1.16 ± 0.00         |
| Y358F    | 3.0 ± 0.5     | 0                   |
| Y426F    | 150.6 ± 13.1  | 1.13 ± 0.01         |
| Y479F    | 103.2 ± 16.5  | 1.72 ± 0.39         |
| Y482F    | 204.0 ± 4.0   | 1.14 ± 0.01         |
| Y486F    | 22.6 ± 0.2    | 0.01 ± 0.00         |
| Y492F    | 135.0 ± 6.7   | 0.61 ± 0.08         |

FIG. 2. Competitive inhibition of carnitine transport by TEA. Rat and human OCTN2 cDNAs were expressed heterologously in HeLa cells. Transport of carnitine was measured for 30 min in the presence of Na⁺ over a carnitine concentration range of 1–75 μM. The concentration of TEA was 0.12 mM in the case of rat OCTN2 and 4 mM in the case of human OCTN2. Only cDNA-specific transport was used in kinetic analysis. V, carnitine transport in pmol/10⁶ cells/30 min; S, carnitine concentration in μM.

FIG. 3. Na⁺ activation kinetics of carnitine transport for wild type rat OCTN2 and the P478L and Y211F mutants of rat OCTN2. The wild type and mutant cDNAs were expressed in HeLa cells. Transport of carnitine (20 μM) was measured at pH 8.5 in the absence of Na⁺. Transport values at maximal activation were normalized for construction of Hill-type plots (inset).
M352R/L352R mutant in that these mutations abolished both carnitine transport and TEA transport. These studies also show that tyrosyl residues are not likely to constitute the Na⁺-binding site in OCTN2.

Transport Function of Cross-species OCTN2 Chimeras—The findings that the P478L mutation and the Y211F mutation are not located in the Na⁺-binding site but still abolish carnitine transport without affecting TEA transport suggest that the carnitine transport site and the TEA transport site may not be identical. However, they may overlap to some extent, providing the basis for the observed competitive interaction between the two substrates. The P478L and Y211F mutations may be located at a site involved only in carnitine transport but not in TEA transport. In contrast, the mutations that abolish carnitine transport as well as TEA transport may be located at a site involved in the transport of both substrates.

To investigate whether there is spatial separation of the sites involved in the transport of carnitine versus TEA, we constructed cross-species OCTN2 chimeras between human OCTN2 and rat OCTN2 and studied their transport function. Human OCTN2 possesses severalfold greater ability to transport carnitine than to transport TEA whereas the ability of rat OCTN2 to transport these two substrates is comparable. As shown in Fig. 4, there was a 51-fold increase in carnitine transport in HeLa cells transfected with human OCTN2 cDNA compared with carnitine transport in vector-transfected HeLa cells. The corresponding increase in TEA transport was 3-fold.

In the case of rat OCTN2, carnitine transport as well as TEA transport increased 13-fold. We used these differential characteristics of rat and human OCTN2s as diagnostic criteria to analyze the transport functions of the rat/human chimeric OCTN2s and identify the domains involved in TEA transport and carnitine transport. Three sets of complementary chimeras were constructed for this purpose with splice junctions at amino acid positions 449/450, 239/240, and 122/123. The first splice site lies in the fifth extracellular loop between transmembrane domains 9 and 10, the second splice site in the 5th transmembrane domain, and the third splice site in the first extracellular loop between transmembrane domains 1 and 2 (Fig. 5). The transport characteristics of these six chimeras are given in Fig. 4. Chimera 1 behaved like human OCTN2, and chimera 2 behaved like rat OCTN2 in carnitine transport function as well as in TEA transport function. This suggests that switching the C-terminal domains (108 amino acids) between the two OCTN2s did not alter the transport function. This domain contains the site of the P478L mutation. Because this mutation affected only the transport of carnitine, this domain is likely to be involved exclusively in carnitine transport. In addition, the function of this domain in facilitating carnitine transport is apparently identical in human OCTN2 and rat OCTN2 because swapping of this domain between the two transporters did not influence their transport characteristics. The findings that the P478L mutation stimulates TEA transport suggest that the mutation might cause conformational changes in the protein in such a way as to facilitate TEA transport.

Chimeras 3 and 4 are very interesting. Chimera 3 functions as human OCTN2 in terms of carnitine transport and as rat OCTN2 in terms of TEA transport. Chimera 4 functions as rat OCTN2 in terms of carnitine transport and as human OCTN2 in terms of TEA transport. These data provide the first indication that the sites responsible for carnitine transport and TEA transport are spatially separated. The region downstream to...
the 239/240 splice junction appears to be involved in TEA transport, and the region upstream to the splice junction appears to be involved in carnitine transport. Chimeras 5 and 6 provide additional evidence for the spatial separation of the transport sites for carnitine and TEA. Chimera 5 functions as rat OCTN2 in terms of carnitine transport and as human OCTN2 in terms of TEA transport. The converse is true with chimera 6. These data show that the region downstream to the 122/123 splice junction is involved in carnitine transport, and the region upstream to the splice junction is involved in TEA transport. Taken together, the results with the chimeric transporters indicate that the TEA transport site consists of the region containing the first 122 amino acids and the region containing the amino acids 240–449. The carnitine transport site consists of the region containing the amino acids 123–239 and the region containing the C-terminal 108 amino acids.

To our knowledge, this is the first example of significant spatial separation of the transport sites for different substrates in an organic solute transporter. This also constitutes the first report of mutations in a transporter that have differential influence on the handling of different substrates. Furthermore, the present findings are of significant clinical relevance because these mutations have been identified in humans with primary carnitine deficiency. Patients with such mutations exhibit impairment in OCTN2-mediated carnitine transport function but are unlikely to have any impairment in OCTN2-mediated organic cation transport function. Similarly, there may be mutations in OCTN2 in humans that abolish the organic cation transport function but leave the carnitine transport function unaffected. Such individuals are not expected to have primary carnitine deficiency but may experience defective handling of organic cations in various tissues.

Acknowledgment—We thank Vickie Mitchell for excellent secretarial assistance.

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