Role of a Hydrophobic Pocket in Polyamine Interactions with the Polyspecific Organic Cation Transporter OCT3*

Dan C. Li, Colin G. Nichols, and Monica Sala-Rabanal

From the Department of Cell Biology and Physiology, and the Center for the Investigation of Membrane Excitability Diseases (CIMED), Washington University School of Medicine, St. Louis, Missouri 63110

Organic cation transporter 3 (OCT3, SLC22A3) is a polyspecific, facilitative transporter expressed in astrocytes and in placental, intestinal, and blood-brain barrier epithelia, and thus elucidating the molecular mechanisms underlying OCT3 substrate recognition is critical for the rational design of drugs targeting these tissues. The pharmacology of OCT3 is distinct from that of other OCTs, and here we investigated the role of a hydrophobic cavity tucked within the translocation pathway in OCT3 transport properties. Replacement of an absolutely conserved Asp by charge reversal (D478E), neutralization (D478N), or even exchange (D478E) abolished MPP⁺ uptake, demonstrating this residue to be obligatory for OCT3-mediated transport. Mutations at non-conserved residues lining the putative binding pocket of OCT3 to the corresponding residue in OCT1 (L166F, F450L, and E451Q) reduced the rate of MPP⁺ transport, but recapitulated the higher sensitivity pharmacological profile of OCT1. Thus, interactions of natural polyamines (putrescine, spermidine, spermine) and polyamine-like potent OCT1 blockers (1,10-diaminodecane, decamethonium, bistrriethylaminodecane, and 1,10-bisquinoicinulidene) with wild-type OCT3 were weak, but were significantly potentiated in the mutant OCT3s. Conversely, a reciprocal mutation in OCT1 (F161L) shifted the polyamine-sensitivity phenotype toward that of OCT3. Further analysis indicated that OCT1 and OCT3 can recognize essentially the same substrates, but the strength of substrate-transporter interactions is weaker in OCT3, as informed by the distinct makeup of the hydrophobic cleft. The residues identified here are key contributors to both the observed differences between OCT3 and OCT1 and to the mechanisms of substrate recognition by OCTs in general.

Background: OCT3 pharmacology is distinct from that of other OCTs, which may relate to non-conserved residues in a putative binding pocket.

Results: Mutating pocket residues to their OCT1 counterparts shifts OCT3 polyamine-like blocker sensitivity toward that of OCT1, and vice versa.

Conclusion: OCT substrate-recognition mechanisms are fine-tuned by the makeup of the binding cleft.

Significance: A mechanism has not yet been proposed.
Molecular Basis of OCT3 Substrate Selectivity

Experimental Procedures

Tested Compounds—Radiolabeled model substrate methyl-[3H]4-phenylpyridinium ([3H]MPP+) iodide (specific activity: 85 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The following compounds were examined in this study (Fig. 4): tetramethylammonium (TMA), TEA; naturally occurring polyamines putrescine, spermidine, and spermine (Spmn); and synthetic polyamine analogues 1,7-diaminoheptane (DA-7), 1,10-diaminocadecane (DA-10), 1,6-bistrimethylaminohexane (TMA-6), decamethonium (TMA-10), 1,6-bistriethylaminohexane (TEA-6), 1,10-bistriethylaminocadecane (TEA-10), bisquinuclidinohexane (Quin-6), and 1,10-bisquinuclidinedecane (Quin-10). All compounds were purchased from Sigma. Novel polyamine analogues TMA-6, TEA-6, TEA-10, Quin-6, and Quin-10 were custom synthesized.

Site-directed Mutagenesis—Rat OCT3 (rOCT3) and human OCT3 (hOCT3) (both in the pSPORT vector) were linearized with NotI and BamHI (New England Biolabs, Ipswich, MA), respectively, and transcribed in vitro using the T7 mMessage mMachine kit (Applied Biosystems, Foster City, CA). Mouse OCT1 (mOCT1) (in pBluescript II-SK) was linearized using XhoI and transcribed using the T7 mMessage mMachine kit (Applied Biosystems, Foster City, CA). Mouse OCT1 (mOCT1) (in pBluescript II-SK) was linearized with NotI and BamHI (New England Biolabs, Ipswich, MA), respectively, and transcribed in vitro using the T7 mMessage mMachine kit (Applied Biosystems, Foster City, CA). Mouse OCT1 (mOCT1) (in pBluescript II-SK) was linearized using XhoI and transcribed using the T7 mMessage mMachine kit (Applied Biosystems, Foster City, CA).

Generation of OCT3 and OCT1 mutants was carried out with a QuikChange site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA). Oligonucleotide primers used for mutagenesis were purchased from Sigma. Primer design and full cDNA sequence analysis were performed using DNASTAR Lasergene Suite 12.2 (DNASTAR, Madison, WI).

Heterologous Expression of OCTs in Oocytes—Mature female Xenopus laevis frogs were purchased from Xenopus Express (Brooksville, FL). All animal protocols followed guidelines approved by the Washington University School of Medicine and the National Institutes of Health. Frogs were anesthetized with a 0.1% tricaine solution buffered with 0.1% NaHCO3 prior to survival surgery in which a portion of the ovary is removed. Stage V-VI oocytes were isolated and maintained at 18 °C in modified Barth’s solution of the following composition: 88 mM NaCl, 2.4 mM NaHCO3, 1 mM KCl, 0.3 mM Ca(NO3)2, 0.4 mM CaCl2, 0.8 mM MgSO4, and 10 mM Hepes/Tris (pH 7.4), and supplemented with 50 mg/liter of gentamicin, 6 mg/liter of ciprofloxacin, and 100 mg/liter of streptomycin sulfate/100,000 units/liter of penicillin G sodium (Life Technologies). Oocytes were injected 1 day after isolation with 25 ng of mOCT1, rOCT3, or hOCT3 cRNA, and maintained at 18 °C.

MPP+ Transport and Competition Assays—Oocytes were incubated in 0.1 μM [3H]MPP+, in buffer with the following composition: 100 mM NaCl, 3 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM Hepes/Tris (pH 7.4). After 30 min, oocytes were rinsed thoroughly with ice-cold buffer of the same composition, individually lysed with 2% sodium dodecyl sulfate, and assayed for radioactivity in a scintillation mixture solution (Econo-Safe, Research Products International, Mount Prospect, IL). [3H]MPP+ uptake was measured in the absence or presence 100 μM MPP+, 1 mM TMA or TEA; 1 or 10 mM putrescine, spermidine, or spermine; or 1 mM DA-7, DA-10, TMA-6, TMA-10, TEA-6, TEA-10, Quin-6, or Quin-10. Dose-response competition assays were performed in presence of 0.1 μM MPP+ (0.025 μM [3H]MPP+) and increasing concentrations of putrescine, spermidine, spermine (0–25 mM), DA-10, TMA-10, TEA-10, Quin-6, or Quin-10. All experiments were performed 5 days after RNA injection and carried out at 20–22 °C. In each experiment, non-injected oocytes from the same preparation served as controls.

Data Analysis—PyMOL (Schrödinger Inc., New York, NY), Accelrys Draw 4.1 (Accelrys Inc., San Diego, CA) and CorelDRAW X7 17.0 (Corel Corporation, Mountain View, CA) were used for protein structure visualization, chemical structure drawing, and figure preparation. All data are shown as the mean ± S.E. for at least three independent experiments from different donor frogs, using 6–10 oocytes per condition. Statistical comparisons were performed with one-way analysis of variance followed by a post hoc Tukey test using GraphPad Prism (GraphPad Software, La Jolla, CA). For the dose-response competition studies, data were fit with Equation 1 (modified from (23)),

\[
J = \frac{J_{\text{app}}(\text{MPP}^+) / K_{\text{app}}}{I} + I
\]

where \(J\) is the rate of MPP+ uptake in the presence of a given concentration of inhibitor \(I\) normalized to the uptake rate in the absence of inhibitors \(J_{\text{max}}\), \(J_{\text{max}} / K_{\text{app}} = J_{\text{max}} / (K_{\text{IC}50} / K_{\text{app}})\), \(K_{\text{IC}50}\) being the apparent affinity constant of MPP+ transport, and \(K_{\text{app}}\) the...
concentration of test compound required to inhibit 50% of \( J_{\text{max}} \); and \( K_{\text{app}} \) is an apparent inhibitory constant for the test compound, defined as \( IC_{50} \) (1\(^{-}\)\( [MPP^{-}] \)/\( K_{0.5} \)). \( [MPP^{-}] \) is much smaller than the estimated \( K_{0.5} \) for MPP\(^{+}\) (100 \( \mu \)M) (15), \( K_{\text{app}} \sim IC_{50} \). SigmaPlot 10.0 (Systat Software, San Jose, CA) was used for nonlinear regression analysis.

**Results**

Sequence alignment of the mouse, rat, and human orthologs of \( \text{OCT1 and OCT3} \) (Fig. 1A) reveal that two of the residues identified as potentially contributing to substrate affinity and/or sensitivity in \( \text{rOCT1} \) (28, 30), namely Phe-161 and Gln-448, are not conserved in \( \text{rOCT1} \) and \( \text{hOCT3} \). Key \( \text{rOCT1} \) residue Phe-160 (32) is conserved in \( \text{OCT3s} \), but adjacent Phe-161 is not. Absolutely conserved Asp-475 has been hypothesized to be the single site for the stabilization of the positive charge of transported substrates. Residues highlighted in A are visualized for \( \text{rOCT1} \) (B) and \( \text{hOCT3} \) (C) using a tridimensional model for \( \text{hOCT2} \) (27, 36) as a template. Conserved residues are in gray, except for the putative Asp binding site, which is in red. Left panels, viewed in the plane of the membrane; right panels, close-up views from the extracellular side showcasing the spatial distribution of the residues investigated.
are in close proximity and form a ring-like structure of amino acid side chains that potentially function as substrate sensitivity determining residues in OCT1 (Fig. 1B) and OCT3 (Fig. 1C).

Effect of Mutations at a Proposed Substrate Binding Site in OCT1 on MPP⁺ Uptake in OCT3—Uptake of 0.1 μM [3H]MPP⁺ into non-injected oocytes and oocytes injected with wild-type (WT) and mutant OCT3 cRNA was assessed (Fig. 2). In oocytes expressing WT rOCT3, MPP⁺ transport rates were ~30-fold higher than in non-injected oocytes (Fig. 2A). Mutations in Asp-473 drastically reduced rOCT3-mediated uptake of MPP⁺ to levels comparable with that of non-injected oocytes, not only for charge neutralization (D473N) or charge reversal (D473R) mutations, but also for charge exchange mutations (D473E) (Fig. 2A). In oocytes expressing WT hOCT3, MPP⁺ transport rates were about 20-fold higher than in non-injected oocytes (Fig. 2B). The same mutations were made for the corresponding aspartate residue in hOCT3 (Asp-478), and D478E, D478N, and D478R similarly abolished hOCT3-mediated transport (Fig. 2B). These results suggest that this aspartate residue (Asp-473 in rOCT and Asp-478 in hOCT3) is an absolute requirement for OCT3-mediated transport and may participate in the stabilization of the positively charged cationic moiety of MPP⁺ in particular, and OCT substrates in general. It has been observed previously that, in OCT1 and OCT2, Asp-475 is critical for affinity and substrate selectivity (28, 35, 37). Additionally, the observed reduction of MPP⁺ uptake in the charge replacement mutation (Asp to Glu), in both OCT3 (Fig. 2) as well as OCT1 and OCT2 (35, 37), further implies that this aspartate residue may not only contribute to an electrostatic stabilization of positively charged substrates, but also that the steric and spatial orientation of this interaction is critical for the transport mechanism of both the rat and human OCT3.

Role of Non-conserved Binding Pocket Residues on MPP⁺ Uptake in OCT3—Next, uptake of 0.1 μM [3H]MPP⁺ into oocytes expressing mutations in rOCT3 and hOCT3 at residues lining a putative substrate binding pocket of the transporter was assessed (Fig. 2). In rOCT3 (Fig. 2A) and hOCT3 (Fig. 2B), mutation of these residues to their corresponding positions in rOCT1 (L161F, F445L, and E446Q in rOCT3; L166F, F450L, and E451Q in hOCT3) decreased MPP⁺ uptake rate. MPP⁺ uptake rates for single point mutations in rOCT3 and hOCT3 at putative binding pocket residues remained at least 10-fold above that of non-injected oocytes (~15–25-fold for rOCT3 mutants and 10–15-fold for hOCT3 mutants) (Fig. 2). Although the MPP⁺ uptake rate decreased in these mutants compared with the wild-type transporter, the remaining OCT3-mediated activity in these mutant transporters was sufficiently above the background to allow for assessment of MPP⁺ uptake inhibition. However, MPP⁺ uptake rates for the double mutants (F445L/E446Q in rOCT3 and F450L/E451Q in hOCT3) or the triple mutants (L161F/F445L/E446Q in rOCT3 and L166F/F450L/E451Q in hOCT3) were less than 5-fold above that of non-injected oocytes (Fig. 2), and therefore these mutants were not considered for competition assays.

Inhibition of MPP⁺ Uptake by Polyamines and Polyamine Analogues—For further insights to the role of non-conserved residues in determining substrate-transporter interactions in OCT3, as compared with OCT1, blocking potency was assessed for the naturally occurring polyamines (putrescine, spermidine, and spermine) along with a series of structural polyamine analogues (Fig. 3). Each of these potential substrates was screened...
for effect on the uptake of 0.1 μM [3H]MPP⁺ into oocytes expressing mOCT1, or WT or mutant OCT3 (Fig. 4). Putrescine, spermidine, and spermine were added to the incubation buffer at 1 and 10 mM, unlabeled MPP⁺ was added at the approximate Kᵦ₀.₅ value (0.1 mM) (15), and all other compounds were tested at 1 mM.

Uptake of [3H]MPP⁺ into oocytes expressing WT and mutant rOCT3 or hOCT3 was inhibited 50% by unlabeled MPP⁺ (Fig. 4A) as expected from the calculated Kᵦ₀.₅ (15). The isolated head group cations, TMA and TEA, did not have any effect on MPP⁺ uptake in WT or mutant OCT3 (Fig. 4, D and E), implying that the mutations did not confer any discernible change in transporter affinity and/or sensitivity for these small monovalent compounds. Consistent with previous reports (15), there was no measurable inhibition of MPP⁺ uptake by the natural polyamines for either rat or human WT OCT3 at either 1 or 10 mM (Fig. 4B). In contrast, mOCT1-mediated MPP⁺ uptake was substantially inhibited by the polyamines at 10 mM, and with the exception of spermidine, even at 1 mM (Fig. 4B, white bars).

L161F, F445L, and E446Q in rOCT3 and L166F, F450L, and E451Q in hOCT3 increased sensitivity to 10 mM putrescine, spermidine, and spermine in rOCT3 and hOCT3, with no obvious differences in blocking potency between mutations (Fig. 4B, color bars). In general, mutations shifted the OCT3 polyamine-analogue blocker sensitivity profile to that of mOCT1, in particular for the decyl compounds DA-10, TMA-10, TEA-10, and Quin-10, but also for the less hydrophobic, shorter chained hexyl and heptyl compounds DA-7, TMA-6, TEA-6, and Quin-6 (Fig. 4, C–F, color bars), all of which significantly block OCT1 (Fig. 4, C–F, white bars) and OCT2 (15). Kinetic analysis with the endogenous polyamines and representative analogues (Fig. 5 and Table 1) revealed that all three mutations strengthen substrate-transporter interactions, as indicated by the generalized leftward shift of the dose-response curves (Fig. 5, A–G), and the corresponding decrease in IC₅₀ values (Table 1), toward those for OCT1. Comparison between the IC₅₀ values for OCT1 and those for OCT3 (Fig. 5H, squares) yielded a linear relationship, suggesting that, at least for this class of chemicals, OCT1 and OCT3 can interact with essentially the same compounds, and that there is a global shift in the strength of substrate-transporter interactions between the two isoforms, rather than a change in selectivity; the slope of this relationship was well below unity (Fig. 5H, dashed line), as expected because polycations and polycation-like compounds are consistently more potent blockers of OCT1 than of OCT3 (Figs. 4 and 5, and Table 1). The mutations did not change the substrate selectivity profile of OCT3, as comparison between mutant and WT IC₅₀ values (Fig. 5H, circles) also yielded linear relationships, but they did increase the strength of substrate/blocker-transporter interactions, as manifested by the <1 slopes of these relationships. Finally, whereas all mutations increased the apparent affinity of OCT3 for polyamines and polycation analogues, neither was able to fully recapitulate the OCT1 phenotype (Figs. 4 and 5, and Table 1).

Effect of Mutations in Non-conserved Residues on OCT1 Transport Properties—To further test the hypothesis that non-conserved residues within the putative hydrophobic pocket account for differences in substrate-transporter interactions between OCT1 and OCT3, we mutated Phe-161, Leu-447, and Gln-448 in mOCT1 to their corresponding residues in OCT3 (Fig. 1) and examined the functional properties of the mutant...
transporters (Fig. 6). L447F and Q448E drastically reduced MPP⁺ uptake rates (Fig. 6A), consistent with previous reports that Leu-447 and Gln-448 are critical for OCT1-mediated transport (29). In F161L-expressing oocytes, MPP⁺ uptake was lower than in the WT, but sufficiently above the background (−10-fold, Fig. 6A) to allow for competition assays (Fig. 6, B–I, and Table 1). Although the interactions with MPP⁺ were seemingly unaffected (Fig. 6A, inset), the apparent affinity for the test polyamine-like compounds was decreased in F161L, as shown by the right-shift of the dose-response curves (Fig. 6, B–H) and the concomitant increase in IC₅₀ values (Table 1). OCT3-to-OCT1 comparison of IC₅₀ values (Fig. 6I) yielded a linear relationship with a slope of −3, and in F161L the blocking potency of the test compounds decreased, which resulted in a mutant to WT IC₅₀ relationship with a slope >1, which is shifted toward the lower sensitivity OCT3 phenotype.
Discussion

The present study addresses two outstanding questions: 1) what is the role of non-conserved residues in the putative OCT3 binding pocket on transporter-substrate interactions; and 2) whether changes at these residues contribute to the observed pharmacological differences between OCT1 and OCT3, in particular with respect to polyamines and structural polyamine analogues. OCT3 is expressed in neurons of various areas of the brain and in glial cells (4, 38), where it may contribute to polyamine-mediated Kir channel modulation and cell-to-cell communication (39, 40). Loss-of-function, overactive, or deregulated Kir channels result in disorders ranging from deafness, epilepsy, and autism, to the systemic Andersen-Tawil, Barter, and EAST/SeSAME syndromes (41–45). Use of OCT3 as a vehicle to deliver high affinity polyamine-like compounds to help alleviate some of these conditions might be a powerful pharmacological tool. Conversely, because OCT3 is expressed in organs of drug absorption, disposition, and excretion, such as the intestine and the blood-brain barrier (1), it might be exploited to enhance the bioavailability of pharmaceuticals aimed at diagnosis or treatment of diseases caused by polyamine imbalance. Polyamine transport inhibitors are being

FIGURE 5. Mutations at putative binding pocket residues shift the polyamine interaction profile of OCT3 toward that of OCT1. A–G, concentration dependence of MPP⁺ uptake block by the natural polyamines and selected polyamine analogues in oocytes expressing mOCT1, or WT or mutant hOCT3. Symbols, uptake of 0.1 μM MPP⁺ (0.025 μM [3H]MPP⁺) was measured in the presence of increasing concentrations of putrescine, spermidine, spermine (0–25 mM), DA-10, TMA-10, TEA-10, or Quin-10 (0–2.5 mM), and results were normalized to the uptake in the absence of test compounds. Data are mean ± S.E. for at least 2 experiments, each with at least 6 oocytes per condition. Lines, data were fit with Equation 1 to estimate the IC₅₀ values (shown in Table 1). F, symbols, IC₅₀ values for mOCT1 or mutant hOCT3 (y axis) were plotted against those for WT-hOCT3 (x axis). Lines, data followed linear relationships (r² = 0.81–0.99) with estimated slopes of 0.23 (mOCT1), 0.34 (L166F), 0.38 (F450L), and 0.25 (E451Q); for reference, a linear relationship with a slope of 1 is shown (black line).
investigated as anticancer drugs (46) and carcinogenesis and tumor growth have been associated with increased intracellular polyamine levels (47) and thus OCT3 might also be targeted for the delivery of cytotoxic polyamine analogues or polyamine-conjugated imaging probes.

A Conserved Aspartate in TM11 is Essential for OCT3 Transport Activity—OCT1 and OCT2 have been well characterized biochemically in numerous mutagenesis studies that have identified an aspartate residue (Asp-475 in rOCT1) in predicted transmembrane helix 11 (TM11) to be critically important for determining affinity and selectivity of substrates in OCT1 and OCT2 (28, 35, 37). The mechanism of substrate recognition and transport in OCT3, on the other hand, is less understood as the set of biochemical data collected in systems expressing OCT3 is, at present, extremely limited. Here, we have shown that this conserved aspartate residue is essential for transport in OCT3. Mutation of this residue in rOCT3 (Asp-478) and in hOCT3 (Asp-478) resulted in complete abolition of carrier-mediated activity (Fig. 2), in support of the hypothesis that this TM11 Asp functions in all OCTs as the singular binding site for the cationic region of transported substrates (32). Although some models of substrate binding have been generated, in particular of spermidine binding to hOCT2 (37), questions regarding the exact role of this conserved residue in substrate recognition persist. In particular, the present study suggests that simply the presence of a negative charge in that position is not sufficient to accomplish substrate binding and translocation, because the substitution of Glu for Asp results in a complete loss of function as well (Fig. 2). Rather, the unique spatial arrangements provided by the specific side chain of Asp in this position may be required for substrate coordination and further stabilization of the substrate positive charge.

A Distinct Binding Pocket Makeup Accounts for Pharmacological Differences between OCT1 and OCT3—Previously we observed that for polyamine analogues, increasing the hydrophobic character of the charged end groups and increasing the acyl chain length between them strengthens interaction with OCT1, and that these requirements are accentuated in OCT3 (15). Here, we show that mutations in non-conserved putative binding pocket residues in rOCT3 or hOCT3 to their OCT1 counterparts, including newly identified Phe-161, confers inhibition of MPP⁺ uptake by putrescine, spermidine, and spermine at 10 mM (Fig. 4B). This gain of polyamine sensitivity in the putative binding pocket mutants thus, at least partially, recapitulates the substrate selectivity profile of OCT1 with respect to natural polyamines. The mutations shifted the competition phenotype of OCT3 toward that of OCT1 for polyamines and polyamine analogues alike, but no single point mutation alone fully recapitulated the pharmacology of OCT1 (Fig. 5 and Table 1). Polyamines and polyamine analogues interacted poorly with the wild-type OCT3s but strongly with the mutants, as they do with OCT1 (Figs. 4 and 5, and Table 1). Conversely, reciprocal mutations in OCT1, in particular F161L, shifted the pharmacological profile toward a less sensitive OCT3-like phenotype (Fig. 6 and Table 1). This illustrates a generalized increase of sensitivity to polyamine blockers in OCT3 mutants (or a generalized decrease in F161L-OCT1) rather than a change in substrate specificity, and suggests that, at least for polyamine-like substrates, OCT1 and OCT3 can interact with the same compounds, but that the interactions at the substrate-binding pocket are stronger in OCT1.

Our analysis is corroborated by qualitative and quantitative comparison between the pharmacological profiles of OCT3 and OCT1. For example, the decyl-polyamine analogue compounds (DA-10, TMA-10, TEA-10, and Quin-10) did not inhibit MPP⁺ uptake in WT OCT3, but decreased it up to 90% in binding pocket mutants (Figs. 4, C–F). The highly hydrophobic Quin-10 was a mild blocker of MPP⁺ uptake in WT OCT3, but in all OCT3 mutants the blocking potency of Quin-10 increased to levels similar to those of OCT1 (Fig. 4F); a similar trend was observed in the decyl-TEA compound, TEA-10 (Fig. 4E), indicating a robust phenotypic shift toward the substrate sensitivity profile of OCT1 in mutants rOCT3 and hOCT3. For hOCT3, the increase in apparent affinity of mutant transporters for these compounds was confirmed by detailed dose-response analysis (Fig. 5 and Table 1). Although a good deal of overlap exists between the range of blocking potencies of shorter chain polyamine analogues (DA-7, TMA-6, TEA-6, and Quin-6) and their decyl counterparts (DA-10, TMA-10, TEA-10, and Quin-10), a general trend of increasing blocking potency can be observed when moving from the shorter to the longer acyl chain analogues within groups (Fig. 4, C–F). In the binding pocket mutants of rat and human OCT3, significant inhibition of MPP⁺ uptake was observed for the short chain TEA-6 and Quin-6, none of which inhibited transport in the WT (Fig. 4, D–F). Notably, this trend was also observed in the substrate selectivity profiles of OCT1 where MPP⁺ uptake was strongly inhibited by the shorter chain polyamine analogues DA-7, TMA-6, TEA-6, and Quin-6 (15).
Each mutation in rat or human OCT3 represents one of many residues that purportedly contribute to determining substrate selectivity in OCTs (21, 22, 28, 29, 32, 34, 35, 37). Therefore, it is unlikely that any one mutant can completely reproduce the blocker potency profile of OCT1, and we cannot discard the possibility that residues other than the ones investigated here play critical roles in shaping OCT3-substrate interactions. Mutations at putative hydrophobic cleft-lining residues (L161F, F445L, and E446Q in rOCT3 and L166F, F450L, and E451Q in hOCT3) shifted the pharmacological profile of rat and human OCT3 toward the higher sensitivity phenotype of OCT1, but no single mutation was sufficient to completely reproduce the trend observed (Figs. 4 and 5, and Table 1). Reciprocal mutations in OCT1, in particular F161L, decreased the apparent affinity of the transporter for these compounds, toward an OCT3-like, low-sensitivity phenotype (Fig. 6 and Table 1). At the same time, each of these mutations reduced the MPP⁺ transport rate (Figs. 2 and 6A), and oocytes expressing...

FIGURE 6. Effect of binding pocket mutations on the transport properties of mOCT1. A, uptake of 0.1 μM [³H]MPP⁺ in non-injected oocytes (Ni, white bars) and in oocytes injected with wild-type (gray bars) or mutant mOCT1 (black bars) cRNA. Inset, uptake of 0.1 μM [³H]MPP⁺ in oocytes expressing WT (gray) or F161L-mOCT1 (black) in the presence of 100 μM unlabeled MPP⁺. Results are mean ± S.E. for 3 experiments from different oocyte preparations, each experiment with at least 8 oocytes per condition. B–H, concentration dependence of MPP⁺ uptake block by the natural polyamines and selected polyamine analogues in oocytes expressing hOCT3, or WT- or F161L-mOCT1. Symbols, uptake of 0.1 μM MPP⁺ (0.025 μM [³H]MPP⁺) was measured in the presence of increasing concentrations of putrescine, spermidine, spermine (0–25 mM), DA-10, TMA-10, TEA-10, or Quin-10 (0–2.5 mM), and results were normalized to the uptake in the absence of test compounds. Data are mean ± S.E. for at least 2 experiments, each with at least 6 oocytes per condition. Lines, data were fit with Equation 1 to estimate the IC₅₀ values (shown in Table 1). Data for hOCT3 and WT-mOCT1 are the same as in Fig. 5. Symbols, IC₅₀ values for hOCT3 or mutant F161L-mOCT1 (y axis) were plotted against those for WT-mOCT1 (x axis). Lines, data followed linear relationships (r² ≥ 0.8) with estimated slopes of 3.4 (hOCT3) and 1.5 (F161L-mOCT1); for reference, a linear relationship with a slope of 1 is shown (black line).
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double or triple mutants registered little or no carrier-mediated activity (Fig. 2), which suggests that these residues are implicated also in substrate translocation. On the other hand, OCT1 and OCT2 have a similar sensitivity profile for polyamines (15) but not for other compounds, such as cimetidine, and in OCT2 the E447Q mutation, equivalent to E446Q in rOCT3 and E451Q in hOCT3, increases the apparent affinity for cimetidine toward an OCT1-like phenotype (27). Together, these results suggest that the set of putative binding pocket-lining residues examined here are at least in part responsible for the observed differences in substrate-transporter interactions between OCT isoforms, which may also provide important insights to the mechanism and structural requirements of OCT substrate recognition and binding.

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