Neutral amino acid transport mediated by ortholog of imino acid transporter SIT1/SLC6A20 in opossum kidney cells

Zorica Ristic, Simone M. R. Camargo, Elisa Romeo, Susana Bodoy, Joan Bertran, Manuel Palacin, Victoria Makrides, Esther M. Furrer, and François Verrey

1Institute of Physiology and Center for Integrative Human Physiology, University of Zürich, Zürich, Switzerland; and 2Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain

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Ristic, Zorica, Simone M. R. Camargo, Elisa Romeo, Susana Bodoy, Joan Bertran, Manuel Palacin, Victoria Makrides, Esther M. Furrer, and François Verrey. Neutral amino acid transport mediated by ortholog of imino acid transporter SIT1/SLC6A20 in opossum kidney cells. Am J Physiol Renal Physiol 290: F880–F887, 2006. First published October 18, 2005; doi:10.1152/ajprenal.00319.2005.—Most neutral L-amino acids are transported actively across the luminal brush-border membrane of small intestine and kidney proximal tubule epithelial cells by a Na+/H+ antiport system named B0 that has been recently molecularly identified (B0AT1, SLC6A19). We show here that the opossum kidney-derived cell line OK also displays a Na+-dependent B0-type neutral L-amino acid transport, although with a slightly differing substrate selectivity. We tested the hypothesis that one of the two B0AT1-related transporters, SLC6A18 (ortholog of orphan transporter XT2) or SLC6A20 (ortholog of the recently identified mammalian imino acid transporter SIT1), mediates this transport. Anti-sense RNA to OK SIT1 (oSIT1) but not to OK XT2 (oXT2) inhibited Na+-dependent neutral amino acid transport induced by OK mRNA injected in Xenopus laevis oocytes. Furthermore, inhibition of oSIT1 gene expression in OK cells by transfection of siRNA and expression of shRNA selectively reduced the Na+-dependent uptake of neutral L-amino acids. Finally, expression of OK cell oSIT1 cRNA in X. laevis oocytes induced besides the transport of the L-imino acid L-Pro also that of neutral L-amino acids. Taken together, the data indicate that in OK cells SIT1 (SLC6A20) is not only an apical imino acid transporter but also plays a major role as Na+-dependent neutral L-amino acid transporter. A similar double role could be envisaged for SIT1 in mammalian kidney proximal tubule and small intestine.

Na+-dependent neurotransmitter transporter family SLC6, in which they form together with the still orphan transporter XT2 (SLC6A18) and rodent XT3 (slc6a20) a phylogenetic cluster (9, 17, 20, 27).

The question of putative additional transporters, next to B0AT1, that transport neutral amino acids across the luminal membrane of small intestine and kidney proximal tubule is still open, as not all cases of neutral amino aciduria (Hartnup disorder) can be explained by mutations in the gene encoding B0AT1 (SLC6A19) (11, 22).

We have shown that the other B0-cluster transporters XT2 and SIT1 are also strongly expressed in the brush-border membrane of small intestine and kidney proximal tubule of mice, although with differential axial gradients compared with B0AT1 (20). Because of their brush-border localization and their high degree of structural identity with B0AT1, we considered XT2 and SIT1 as possible candidate additional transporters of B0-type for which we hypothesized somewhat differential neutral amino acid selectivities.

In the present study, we characterize the B0-type neutral amino acid uptake in opossum kidney proximal tubule cells (OK cells) and test the possible role played by endogenous XT2 and SIT1 for this transport. The observed Na+-dependent, broad-range neutral L-amino acid transport resembles, but does not match, that described for mammalian B0AT1. Using RNA silencing techniques in OK cells and X. laevis oocyte expression, we show here that the B0-like transport of OK cells is mediated, at least in part, by the orthologue of the mammalian IMINO system transporter SIT1 (SLC6A20).

EXPERIMENTAL PROCEDURES

Cell culture. The established cell line from opossum kidney (Didelphis virginiana) (13), clone 3B/2 (kindly provided by Dr. N. Hernando, Zurich) were cultured at 37°C and 5% CO2 in DMEM/Ham’s F-12 medium (1:1) (Life Technologies, Basel, Switzerland) supplemented with 10% heat-inactivated FCS, 5 × 10−5 U/l penicillin, 50 mg/l streptomycin, 2 mM L-glutamine, and 20 mM HEPES as described (19). Experiments were performed at passage 20 to 30.

Uptake experiments in OK cells. OK cells were seeded on plastic dishes (9.6 or 3.8 cm2, Corning Costar, Bodenheim, Germany) at 90–100% confluence and cultivated for 6–7 days. Before the uptake, the cell monolayer was washed five times within 1 min with uptake buffer at 37°C. Uptake buffer with 137 mM NaCl (to measure total transport) or 137 mM N-methyl-D-glucamine (NMDG: for Na+-independent transport) contained also (in mM): 10 HEPES, pH 7.4, 1 CaCl2, 5 KCl, 1 MgCl2, and 10 glucose. Buffer (0.5 or 0.2 ml) containing L-amino acid (L-AA) at the indicated concentration and the

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IN THE LAST TWO decades many amino acid transporters have been molecularly identified and their transport function characterized using heterologous expression in cultured cells or Xenopus laevis oocytes (2, 18, 25, 26). For example, the identifications of gene products corresponding to system B0 (broad scope Na+-dependent neutral amino acid transport) and to system IMINO (Na+-dependent imino acid transport) have been recently reported (1, 11, 22, 24). These two transporters are highly expressed in kidney proximal tubule and small intestine. Functionally they have been characterized in the X. laevis expression system and named according to their amino acid transport characteristics B0AT1 (SLC6A19) and SIT1 (SLC6A20, XT3s1 in rodents) (Table 1) (1, 3, 11, 12, 22, 24). They are structurally members of the Na+/H+ antiport family.

* Z. Ristic and S. M. R. Camargo contributed equally to this work.
Address for reprint requests and other correspondence: F. Verrey, Institute of Physiology, Univ. of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland (e-mail: verrey@access.unizh.ch).

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Table 1. Opossum and mammalian transporters of the SLC6 family B0-cluster

<table>
<thead>
<tr>
<th>Ortholog Names and Levels of Identity with OK Cell Transporters</th>
<th>OK Cells (Didelphis virginiana)</th>
<th>Opossum (Monodelphis domestica)</th>
<th>Human</th>
<th>Mouse</th>
</tr>
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<tbody>
<tr>
<td>SIT1</td>
<td>O8767/2</td>
<td>SLC6A19</td>
<td>slc6a19</td>
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<tr>
<td>SIT2</td>
<td>O8768</td>
<td>SLC6A18</td>
<td>slc6a18</td>
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*Accession numbers: B0AT1 of OK cells DQ155665, human AY_596807, and mouse AJ_633679. SIT1 of OK cells DQ155663, human NM_020208, mouse SIT1 NM_139142, and XT3 NM_011731. XT2 of OK cells DQ155664, human NM_182632, and mouse NM_182632.

For expression in X. laevis oocytes the opossum B0AT1 and SIT1-KSM plasmids were linearized with XhoI and BamHI and 10 mM BCI. For amino acid or Na+ concentration dependence experiments, five 1-Phe or 1-Ile concentrations or eight Na+ concentrations (NMDG substitution) were used. Curves corresponding to the Michaelis-Menten equation were fitted to the data by nonlinear regression, and transport kinetic parameters (Vmax and Km) were derived using Prism software (GraphPad Prism Version 4.0, GraphPad, San Diego, CA).

Transient transfection of OK cells with small-interference RNA. Approximately 12–24 h before transfection, OK cells were plated on plastic dishes (9.6 or 3.8 cm2) and cultured in complete medium without antibiotics until they reached ~90% confluency for the transfection (4, 5). Transient transfections with 70 nM small-interference (si)RNA or 1 µg short-hairpin (sh) RNA construct were performed according to the manufacturer’s instructions (Gene Silencer siRNA transfection reagent, Gene Therapy Systems, San Diego, CA or Lipofectamine 2000, Invitrogen, Carlsbad, CA). siRNA duplex mixed with 4 µl gene silencer reagent and diluted in 1 ml of medium without FCS and shRNA constructs mixed with 4 µl lipofectamine and diluted in 1 ml OPTI-MEN medium were applied to the cell monolayer. Transfection medium was replaced by complete medium after 24 h, and the cells were assayed for Na+-dependent 1-Phe uptake 48 h after transfection in the case of siRNA or 72 h in the case of shRNA constructs. Transfection efficiency of shRNA constructs was monitored by viewing enhanced green fluorescence protein fluorescence using an inverted fluorescence microscope (Nikon ECLIPSE TE 300/200).

Selection and production of oSIT1 si- and shRNAs. The siRNA sequences used were designed (http://websoft2.unige.ch/sciences/biologie/bielle/RNAi.html) and synthesized according to Donzé and Picard (6). The 21-base small double-strand RNAs from oSIT1 were named according to the position of their first base relative to the first ATG: siRNA 165, siRNA 234, siRNA 424, and siRNA 701. Desalted DNA oligonucleotides were ordered from Microsynth (Balgach, Switzerland). For transcription reactions, 1 nmol of each oligonucleotide was annealed with the T7 sense oligonucleotide in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) by heating at 95°C for 2 min and allowing cooling down slowly. The dsDNA was used as template for RNA synthesis with a T7 transcription kit according to the manufacturer’s protocol (MEGAscript, Ambion, Austin, TX). The complementary ssRNA products were annealed (95°C for 5 min followed to 1 h at 37°C), purified, diluted in RNase free water, and used to transfect the OK cells. Negative control OK cells were similarly exposed to the transfection reagent but in the absence of siRNA.

For the shRNA approach, the choice of the oligonucleotide sequences and their annealing and ligation to the vector were performed according to the kit manufacturer (Stratagene, La Jolla, CA). The 29-bp oligonucleotide sequences used were also named according to the position of their first base: siRNA 250, siRNA 420, siRNA 590, and siRNA 830 and ordered from Microsynth. After annealing in 50 mM NaCl with 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA for 3 min and cooling to 37°C, the complementary oligos were ligated to shRNA vector PG-E2hrGFP II (Stratagene) digested with BamHI and XbaI (Promega, Madison, WI). These shRNA DNA constructs were used to transfect OK cells. The negative control for the experiments was a construct containing a scramble sequence distributed by the manufacturer.

Amplification of the OK cell oXT2 and oXT3 sequence. Total RNA from OK cells was isolated with RNAeasy Mini Kit according to the manufacturer (Qiagen, Hombrechtikon, Switzerland). One microgram of total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) and the oligos 5′-CDS and SMART II A, provided with a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). First-strand cDNA was used as a template for 5′-RAE PCR. The SMART RACE cDNA Amplification Kit (Clontech) was used following the manufacturer’s instructions. The specific primers 5′-CTT CCC GCT GGA TTG CTG CTG G-3′ for oXT2 and 5′-CTG GGT GGC TGC TTT GGT CTG G-3′ for oXT1 were used. Nested PCR was performed using 5% of the first PCR volume with specific nested primers 5′-CGC TCC AGG TAG AGT CAG TGT GAG TCC TCG-3′ for oXT2 and 5′-GGC CAT TGG TGG CTC CAT GCA GGG-3′ for oSIT1/XT3. The initial amplification of oXT2 and oSIT1 was exposed to the transfection reagent but in the absence of siRNA. The PCR products were then cloned in the vector pGEMt (Promega) and sequenced using the ABI PRISM BigDye Terminator 3.1 system (Applied Biosystems) with commercial primers. The inserts were subcloned in a pBlueScript modified X. laevis expression vector (KSM) containing both the 5′ and 3′ UTRs of the β-globin gene (kindly provided by Dr. L. V. Virkki). For expression in X. laevis oocytes the opossum B0AT1 and SIT1-KSM plasmids were linearized with XbaI (Promega) and used as template for RNA synthesis from the T3 promoter (mMESSAGE mMACHINE, Ambion). Multiple amino acid sequence comparisons were made using Clone Manager Professional suit (Scientific & Educational Software, Cary, NC).

Transport studies in X. laevis oocytes. Expression studies and uptake measurements in X. laevis oocytes were performed as described previously (15). Briefly, oocytes were injected with the indicated RNA and incubated for 72 to 144 h at 16°C in ND96 buffer (in mM: 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.4, adjusted with Tris, supplemented with 50 mg/l tetracycline). Before uptake measurements, oocytes were preincubated for 2 min at 22°C, and the respective uptake buffer containing amino acids (0.02 µCi/ml) was added. Uptake was stopped after 6 or 30 min by washing the oocytes five times with 3 ml of ice-cold buffer. Oocytes were then distributed to individual vials, lysed in 2% SDS, and the radioactivity was determined by liquid scintillation. As for cells, total uptake was measured in buffer containing NaCl (100 mM), Na+ independence.
was measured in buffer containing NMDG-Cl (100 mM), and in both conditions the buffer contained (in mM): 2 KCl, 1 MgCl₂, 1.8 CaCl₂, and 10 HEPES, pH 7.4. Adjusted with Tris. Glucanate (Glc) salts were used when chloride was substitute; the solution was also buffered with HEPES-Tris, pH 7.4. Concentration dependence of L-Ile and L-Pro uptake was measured for 6 min, and selectivity measurements were performed with 1 mM L-amino acids for 30 min. For competition experiments, the uptake of 0.1 mM L-Pro or L-Ile was performed in the absence (control) or presence of 40 mM cold L-Ile or 2 mM L-Pro, MeAIB, or BCH. The transport in the presence of the inhibitor was normalized to that of L-Pro or L-Ile alone (control) and expressed as percentage.

Hybrid depletion. The oligonucleotides were designed within the amplified sequences of oXT2 and oSIT1 (http://scitools.idtdna.com/AntiSense/), and named according to the position of their first base (related to the first ATG): AS-S 446–466 and 881–900 for oXT2, and AS-S 71–89 and 222–242 for oSIT1. Total RNA was extracted from OK cells using TRIZol reagent (Life Technologies, Basel, Switzerland), and poly(A)⁺ mRNA prepared with PolyATract® mRNA Isolation Systems (Promega) following the manufacturer’s protocol. Oocytes were injected with poly(A)⁺ mRNA (25 ng/oocyte) or not injected. Hybrid depletion experiments were performed as described previously (16). OK cell poly(A)⁺ mRNA was denatured at 65°C for 5 min in the presence of the sense or antisense oligonucleotide complementary to opossum oXT2 or oSIT1 (100 ng oligonucleotide in 50 mM NaCl, RNase free) and further incubated at 42°C for 30 min. The oocytes were injected with poly(A)⁺ mRNA alone or depleted (25 ng/oocyte) and incubated for 72–144 h. Na⁺-dependent transport was calculated and normalized to the poly(A)⁺ mRNA alone and to its respective sense control.

Statistics. All data are shown as means ± SE, and n is the number of cell monolayer dishes or number of oocytes. Experimental protocols were repeated at least twice. Comparison of means was done by t-test with a Mann-Whitney U-test as a nonparametric posttest (P < 0.05), Wilcoxon Signed Rank Test against a hypothetical value, or ANOVA with Dunnett’s test as a posttest (P < 0.05).

RESULTS

OK cells exhibit system B²-type neutral amino acid transport. L-Amino acid transport was assayed in confluent OK cells monolayers grown on plastic dishes in the presence and absence of Na⁺. Under these culture conditions, OK cells differentiate into a polarized phenotype with delineated apical and basolateral membranes (8). Total transport of L-amino acids (in the presence of Na⁺) was always larger than Na⁺ independent transport (in the absence of Na⁺) and the difference between these values was considered as the Na⁺ dependent part of the transport. Na⁺ dependent L-Phe (5 mM) uptake being linear for ~1 min (data not shown), further amino acid uptake experiments were performed for 20-s periods, in particular also for the determination of the substrate concentration dependence of L-Phe and L-Ile (Fig. 1A). Na⁺ dependent apparent affinities (K₀.₅) derived from the fitted Michaelis-Menten curves amounted to 1.9 and 1.1 mM for L-Phe and L-Ile, respectively. Furthermore, replacement of Na⁺ with NMDG or Li⁺ showed that these substitutions are not tolerated (Fig. 1B). Also, the Na⁺ concentration dependence of L-Ile uptake (5 mM) was measured by replacing Na⁺ with NMDG and the derived K₀.₅ for Na⁺ amounted to 2.4 mM (Fig. 1C).

The selectivity of the Na⁺ dependent amino acid uptake of OK cells was assayed for almost all proteogenic L-amino acids, and the uptake rates are shown normalized to that of L-Phe (12.27 ± 0.68 nmol·dish⁻¹·20 s⁻¹; Fig. 1D). Only neutral L-amino acids were found in these conditions to be transported in a Na⁺ dependent manner and the most efficiently transported ones were those with branched side chains followed by aromatic side chains and L-alanine.

To test whether the observed Na⁺ dependent uptake of the different amino acids was potentially via the same transporter(s) as the uptake of L-Phe and to gain some information about relative substrate affinities, uptake of L-Phe (5 mM) was competed by a fivefold excess (25 mM) of the different L-amino acids (Fig. 1E). As expected from the uptake experiments, L-Phe transport was most efficiently inhibited by branched chain amino acids (% inhibition: L-Ile 88, L-Val 86, L-Leu 77) and then by aromatic L-amino acids and L-alanine (L-Phe 67, L-Trp 41, L-Tyr not tested, L-Ala 56). From the other neutral L-amino acids that were shown to be transported relatively well (L-Gln, L-Asn, and L-Thr), only L-Gln competed efficiently for Na⁺ dependent L-Phe transport (% of inhibition: L-Gln 72, L-Asn 14, L-Thr 3). These results suggest that the branched chain and aromatic amino acids as well as L-Ala and L-Gln are to a large extent transported via the same Na⁺ dependent transporter(s) as L-Phe. In contrast, it appears that L-Asn and L-Thr could be transported by other Na⁺ dependent pathways.

A surprising observation for which we have yet no explanation is the fact that the presence of 25 mM Gly stimulated the Na⁺ dependent uptake of L-Phe. It is conceivable that this effect might be indirectly due to the functional interaction of Gly with other transport systems of OK cells rather than to a direct effect at the level of L-Phe transporters. Another surprising observation is the fact that amino acids that were only little or not transported Na⁺ dependently inhibited L-Phe uptake. This was to some extent the case for L-Arg (30%) and L-Pro (34%) and more strikingly for L-Cys, L-His, and L-Met (83, 71, and 85%). Finally, the two tested amino acid analogs BCH and MeAIB did not compete substantially with Na⁺ dependent L-Phe uptake. Based on these results that revealed L-Ile as the most efficient substrate for Na⁺ dependent transport and competitor for L-Phe uptake, we used this branched amino acid as leading substrate for further functional experiments.

OK cell oXT2 and oSIT1 have a high degree of identity with their mammalian orthologs. To test the role of the opossum oXT2 and oSIT1 gene products in the Na⁺ dependent transport of neutral L-amino acids in OK cells, corresponding cDNAs were first amplified from OK mRNA by RACE PCR using degenerate primers based on mammalian XT2 and SIT1 nucleotide sequences. The cDNA open reading frames isolated by this method were 1,854 and 1,782 nucleotides, respectively, and the translated proteins predicted to have 618 and 594 amino acids each. During the course of this study, opossum Monodelphis domestica genome project sequences corresponding to XT2 and SIT1 (XT3) appeared in the Ensembl database (http://www.ensembl.org/). The protein encoded by our oXT2 cDNA obtained from the opossum D.virginiana OK kidney cell line is 95% identical to that encoded by M. domestica entry Q96N87 (gene product #5) and our OK SIT1 (oSIT1) is 96% identical to entry 08876 (gene product #2). oXT2 and oSIT1 share 45% identical residues, whereas their identity with mammalian orthologs amounts to 70 and 85%, respectively (Table 1).
Inhibition of oSIT1 gene expression reduces Na\(^+\)-dependent transport of L-Ile in X. laevis oocytes and OK cell monolayers. As a first experiment to test the putative role of oXT2 and oSIT1 in the Na\(^+\)-dependent transport of neutral L-amino acids observed in OK cell monolayers, the uptake of L-Ile was assayed in X. laevis oocytes expressing poly(A)\(^+\)RNA isolated from intact OK cell monolayers with and without hybrid depletion of oXT2 or oSIT1 mRNA. Poly(A)\(^+\)RNA of OK cells induced L-Ile transport, ~50% of which was Na\(^+\) dependent (Fig. 2A). The hybrid depletion of oXT2 or oSIT1 mRNA from OK cell poly(A)\(^+\)RNA was attempted using 19- to 21-base-long antisense oligonucleotides (AS) and their corresponding sense (S) oligonucleotides as controls. Figure 2B shows that oSIT1 mRNA depletion, and not oXT2 mRNA depletion, decreased the Na\(^+\)-dependent L-Ile transport induced in X. laevis oocytes (65.5 and ~0.3% inhibition, respectively).

To test more directly in OK cell monolayers the putative role of oSIT1 for the Na\(^+\)-dependent transport of neutral amino acids, Na\(^+\)-dependent L-Ile uptake was assayed in X. laevis oocytes expressing poly(A)\(^+\)RNA isolated from intact OK cell monolayers with and without hybrid depletion of oSIT1 mRNA. Poly(A)\(^+\)RNA of OK cells induced L-Ile transport, 50% of which was Na\(^+\) dependent (Fig. 2A). The hybrid depletion of oXT2 or oSIT1 mRNA from OK cell poly(A)\(^+\)RNA was attempted using 19- to 21-base-long antisense oligonucleotides (AS) and their corresponding sense (S) oligonucleotides as controls. Figure 2B shows that oSIT1 mRNA depletion, and not oXT2 mRNA depletion, decreased the Na\(^+\)-dependent L-Ile transport induced in X. laevis oocytes (65.5 and ~0.3% inhibition, respectively).
acids, endogenous oST1 expression was inhibited using two RNA interference methods. On the one hand, in vitro synthesized siRNA was directly transfected into OK cell monolayers and, on the other hand, OK cells were transfected with a plasmid that controls the production of shRNA. Four different siRNAs and four different shRNA constructs were transiently transfected into confluent OK cell monolayers. Figure 2 shows the inhibition of Na\textsuperscript{+}-dependent l-Ile uptake measured with one of the oligonucleotides from each method, namely, with siRNA 424 in Fig. 2C and with shRNA 420 in Fig. 2D. Actually, all four siRNA reduced Na\textsuperscript{+}-dependent l-Ile uptake between 55.68 ± 24.85 and 92.59 ± 14.46% (siRNA 424) relative to their control. Similarly, but to a lesser extent, two shRNA constructs reduced Na\textsuperscript{+}-dependent l-Ile transport 25.34 ± 17.11 and 61.3 ± 12.47%, in the case of shRNA 420. The fact that the Na\textsuperscript{+}-dependent transport of inorganic phosphate was not affected by shRNA 420 expression (P\textsubscript{t} transport in control cells: 2.48 ± 0.23 nmol·9.6 cm\textsuperscript{2} dish\textsuperscript{-1}·10 min\textsuperscript{-1}, with shRNA 420: 2.85 ± 0.14 nmol·9.6 cm\textsuperscript{2} dish\textsuperscript{-1}·10 min\textsuperscript{-1}, n = 3) essentially excludes the possibility that shRNA 420 interferes with a more general mechanism that would affect apical Na\textsuperscript{+}-dependent transports. In summary, the results of all three experimental series with different antisense oligonucleotides and RNA interference approaches strongly suggest that oST1 but not oXT2 plays an important role in the Na\textsuperscript{+}-dependent neutral amino acid uptake in OK cell monolayers.

**DISCUSSION**

Characterization of neutral l-amino acid transport in polarized OK cells. In this study, we first characterized the Na\textsuperscript{+}-dependent transport of l-amino acids in polarized OK cell monolayers, a much used kidney proximal tubule cell model (8). OK cells have been shown previously to functionally express several proximal kidney tubule amino acid transporters, as for instance the apical and basolateral heterodimeric
exchangers B₀⁺-AT-rBAT and LAT2–4F2hc (7). They were also shown earlier to display an apical Na⁺-dependent, electronegic transport of some neutral L-amino acids (L-Phe, L-Ala, L-Val, and L-Gln) (21). Here, we demonstrate that they display a B₀-type transport with apparent affinities (K₀.5) for L-Phe and L-Ile in the lower millimolar range (1.9 and 1.1 mM, respectively; Fig. 1A). This Na⁺-dependent transport showed an apparent Na⁺ affinity of 2.4 mM and a Hill coefficient of 1.00 ± 0.096, suggesting a 1:1 Na⁺-L-amino acid cotransport (Fig. 1, B and C). Although this transport exhibits a broad substrate selectivity range as expected for system B₀ (Fig. 1D), it differs to some extent from the substrate selectivity previously determined for mouse and human B₀AT1, suggesting that (an)other transporter(s) besides B₀AT1 might be involved. The hypothesis that more than a single transporter is involved in Na⁺-dependent neutral amino acid uptake in OK cell monolayers is supported by the complex competition pattern of L-Phe uptake by various L-amino acids. In particular, whereas most substrates displaying an efficient Na⁺-dependent transport also efficiently competed with L-Phe transport, some well-transported substrates did not compete efficiently and some poorly transported substrates competed quite efficiently (Fig. 1E). Additionally, neither the amino acid analog BCH, known to compete with amino acid transport via system L, B₀⁺, and B₀, nor MeAIB, known to compete transport via SIT, inhibited the Na⁺-dependent uptake of L-Phe in OK cells (1, 14, 23, 24). Many of these discrepancies could be explained by the presence of multiple transport systems with overlapping and complementary substrate selectivities and affinities that would be expressed in OK cells. In summary, OK cell monolayers display a B₀⁺-type Na⁺-dependent neutral amino acid transport that is suggested to be mediated by more than one B₀-like transporter, a possibility that might also be considered for mammalian kidney proximal tubule and small intestine.
Hybrid depletion and RNA silencing suggest a major role for oSIT1 in Na\textsuperscript{+}-dependent L-Ile transport in OK cells. After having verified that orthologs of all three SLC6 family B\textsuperscript{0}
cluster transporters B\textsuperscript{0}AT1 (SLC6A19), XT2 (SLC6A18), and SIT1 (SLC6A20) are expressed in OK cells by RT-PCR using degenerate primers (data not shown), we investigated the putative role of both oXT2 and oSIT1 in the transport of neutral L-amino acids. Using as a first approach exogenous OK cell mRNA expression in X. laevis oocytes, coupled with oligonucleotide-mediated hybrid depletion, we obtained results that suggested a role for oSIT1 but not for oXT2 in Na\textsuperscript{+}-dependent neutral amino acid uptake. A similar conclusion was suggested by the use of two RNA interference approaches directly in OK cell monolayers. All three approaches suggested that a very high fraction of the Na\textsuperscript{+}-dependent L-Ile uptake in OK cells is mediated by oSIT1. It is, however, difficult to quantify precisely this fraction due to several reasons. First, we focused only on the “leading substrate” L-Ile. Second, we used it at a high concentration (5 mM) that favors high-affinity/low-affinity transporters like B\textsuperscript{0}AT1 and SIT1 over high-affinity and low-capacity ones. Third, the absence of competitors is nonphysiological, and, fourth, the inhibition of oSIT1 gene expression was not monitored at the protein level (lack of anti-oSIT antibody). Despite these technical limitations, the fact that three different approaches led to the same conclusion strongly supports the hypothesis that oSIT1 plays a major role for the Na\textsuperscript{+}-dependent apical uptake of neutral amino acids in OK cells. In view of the similarity of OK cell oSIT1 and mammalian SIT1 and of the localization of SIT1 in small intestine and kidney proximal tubule, it can be proposed that SIT1 also could play an important role as additional L-amino acid uptake pathway besides B\textsuperscript{0}AT1 in mammalian kidney and intestine.

OK cell oSIT1 is a relatively high-affinity transporter for L-proline and a lower-affinity transporter with higher capacity for neutral L-amino acids. We have called the SLC6A20 ortholog of OK cells oSIT1, because of its similarity with human SIT1. In the evolution of rodents, an additional duplication of this gene leading to a tandem arrangement has taken place and the products of these two genes are provisionally called XT3 and XT3\textsubscript{s1} (Table 1) (9, 10). It should be noted that SIT1 ortholog of OK cells is mediated by oSIT1. In view of the similarity of OK cell oSIT1 and mammalian SIT1, it can be proposed that SIT1 also could play an important role as additional L-amino acid uptake pathway besides B\textsuperscript{0}AT1 in mammalian kidney and intestine.

The functional characterization of oSIT1 in X. laevis oocytes shows that this transporter has characteristics that highly resemble those of mammalian SIT1 (Fig. 3). Indeed, mammalian oSIT1 from human, mouse, and rat was recently functionally characterized and shown to transport l-Pro with a relatively high apparent affinity of \(\sim 200 \mu M\) and also to transport related compounds such as MeAIB, hydroxyproline, methionine, proline, and p-proline (12, 24). Based on these transport characteristics and on the mRNA tissue distribution, this transporter was identified as the previously functionally described system IMINO. The two involved laboratories also reported that the transport of L-Pro by SIT1 was to some extent competed by neutral L-amino acids but that these were not efficiently transported.

We characterized the transport of L-neutral amino acids by oSIT1 in more details, always comparing it with the transport of the imino acid l-proline. Importantly, the apparent affinity (\(K_{0.5}\)) of L-Ile was 10-fold higher (2.6 mM) than that of L-Pro (0.25 mM) (Fig. 3A), a difference that can explain why neutral L-amino acid transport was not investigated further in the first studies on mammalian SIT1. Indeed, when we compared the transport rate of L-Pro with that of neutral amino acids at a similar low concentration (100 \(\mu M\)), L-Pro transport was \(-3.5\)-fold higher. However, it has to be noted that the \(V_{\text{max}}\) of SIT1 is about threefold higher for L-Ile than for L-Pro and that its apparent affinity for L-Ile is similar to that of the dedicated neutral amino acid transporter B\textsuperscript{0}AT1. Direct competition experiments performed in oocytes expressing oSIT1 confirmed that L-Pro and L-Ile are transported by the same transporter. This was confirmed by the fact that MeAIB competed both L-Ile and L-Pro uptake.

At first sight, these latter functional results and the little Na\textsuperscript{+}-dependent L-Pro transport measured in OK cell monolayers (Fig. 1D) do not appear to support the RNA interference-based hypothesis that a large fraction of Na\textsuperscript{+}-dependent L-neutral amino acid transport of OK cells is mediated by oSIT1. An explanation for this apparent discrepancy is that the Na\textsuperscript{+}-independent transport of L-Pro was very high in OK cell monolayers at the elevated substrate concentrations tested (5 mM), whereas the \(V_{\text{max}}\) of oSIT1 for Na\textsuperscript{+}-dependent L-Pro transport is quite low, compared with its \(V_{\text{max}}\) for L-Ile (Fig. 3A) Together, these observations can explain why in our experimental conditions the Na\textsuperscript{+}-dependent transport of L-Pro into OK cells was to a large extent masked.

In summary, the present results demonstrate that oSIT1, and by extrapolation probably also its mammalian orthologs, transports neutral L-amino acids besides L-imino acids. This suggests the possibility that SIT1, besides efficiently (re)absorbing L-Pro, also participates next to B\textsuperscript{0}AT1 to the Na\textsuperscript{+}-dependent (re)absorption of neutral L-amino acids in the kidney proximal tubule and in the small intestine.

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