Identification and functional characterization of a novel human and rat riboflavin transporter, RFT1

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Submitted 11 January 2008; accepted in final form 10 July 2008

Yonezawa A, Masuda S, Katsura T, Inui K. Identification and functional characterization of a novel human and rat riboflavin transporter, RFT1. Am J Physiol Cell Physiol 295: C632–C641, 2008. First published July 16, 2008; doi:10.1152/ajpcell.00019.2008.—Absorption of riboflavin is mediated by transporter(s). However, a mammalian riboflavin transporter has yet to be identified. In the present study, the novel human and rat riboflavin transporters hRFT1 and rRFT1 were identified on the basis of our rat kidney mRNA expression database (Horiba N, Masuda S, Takeuchi A, Saito H, Okuda M, Inui K. Kidney Int 66: 29–45, 2004). hRFT1 and rRFT1 cDNAs have an open reading frame encoding 448- and 450-amino acid proteins, respectively, that exhibit 81.1% identity and 96.4% similarity to one another. In addition, an inactive splice variant of hRFT1, hRFT1sv, was also cloned. The hRFT1sv cDNA, which encodes a 167-amino acid protein, retains an intron between exons 2 and 3 of hRFT1. Real-time PCR revealed that the sum of hRFT1 and hRFT1sv mRNAs was expressed strongly in the placenta and small intestine and was detected in all tissues examined. In addition, hRFT1 and hRFT1sv were expressed in human embryonic kidney (HEK)-293 and Caco-2 cells. HEK-293 cells transfected with green fluorescent protein-tagged hRFT1 and rRFT1 exhibited a fluorescent signal in the plasma membrane. Overexpression of hRFT1 and rRFT1, but not hRFT1sv, increased the cellular accumulation of [3H]riboflavin. The transfection of small interfering RNA targeting both hRFT1 and hRFT1sv significantly decreased the uptake of [3H]riboflavin by HEK-293 and Caco-2 cells. Riboflavin transport is Na+ dependent, and pH independent. Kinetic analyses demonstrated that the Michaelis-Menten constants for the uptake by HEK-293 and Caco-2 cells were 28.1 and 63.7 nM, respectively. We propose that hRFT1 and rRFT1 are novel mammalian riboflavin transporters, which belong to a new mammalian riboflavin transporter family.

RIBOFLAVIN, a water-soluble vitamin also known as vitamin B2, is essential for normal cellular functions. Its most important biologically active forms, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), act as intermediaries in the transfer of electrons in biological oxidation-reduction reactions. Under conditions of physiological and pathological stress, humans are susceptible to developing riboflavin deficiency. Such a deficiency in pregnancy and adolescence induces developmental abnormalities and has been implicated as a risk factor for anemia, cancer, cardiovascular disease, and neurodegeneration (32).

Humans are unable to synthesize riboflavin and thus acquire it as a nutrient. In vivo, in situ, and membrane vesicle experiments have elucidated the predominant role of carrier-mediated absorption in the intake of riboflavin by the small intestine and colon (4, 7, 17, 21, 24, 34, 41, 44). In addition, the subject of riboflavin excretion has gained increasing attention. Jusko et al. (18, 19) and others (7, 25, 40, 45) demonstrated saturable renal reabsorption and accumulation of riboflavin. Therefore, riboflavin transporters are thought to be essential for the maintenance of riboflavin homeostasis in the intestine and kidney. Since the mid 1990s, the mechanism by which riboflavin is transported has been examined using several human-derived cell lines (14, 15, 20, 23, 36–38). These studies indicated that the cellular uptake of riboflavin was saturable, suggesting that the transporter(s) was (were) intrinsically expressed in these cells. However, a mammalian riboflavin transporter has yet to be identified.

We previously constructed an mRNA expression database by sequencing cDNA clones randomly selected from a rat kidney cDNA library (12). Analysis of this database revealed that 16.7% of its 2,048 genes were of unknown function. As transporter mRNA accounts for 3.7% of the genes of known function in this database, at least 15 novel transporters could be included among the genes of unknown function. We successfully identified a Na+-dependent d-glucose/d-fructose transporter 1, NaGLT1, which mediates tubular reabsorption of d-glucose and d-fructose (11, 13).

In the present study, we searched for a novel transporter among the functionally unknown genes of this database and identified a human and rat riboflavin transporter (hRFT1 and rRFT1, respectively). An inactive spliced variant of hRFT1, hRFT1sv, was also cloned. The tissue distribution, cellular localization, and functional characterization of these transporters were examined.

MATERIALS AND METHODS

Isolation of rat and human riboflavin transporter 1. We previously constructed a rat kidney mRNA expression database by sequencing cDNA clones randomly selected from a kidney cDNA library (12). Using the SOSUI program (10), we searched among the 2,048 clones of this database for uncharacterized multitransmembrane proteins. One clone encoding a 10-transmembrane protein (GenBank accession no. XM_001075182, similar to G protein-coupled receptor 172B) was identified. On the basis of this sequence, a 5'–RACE-PCR were carried out with the reverse primers 5'-TCT-3' and the forward primers 5'-TTCTTTTGTTGTTGACGG-3'. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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RACE fragments were ligated, cloned into the plasmid vector pBK-CMV (Stratagene, La Jolla, CA) or pEGFP-C1 (Clontech), and sequenced using a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan).

In addition, we searched for the human ortholog in the GenBank database by using the TBLASTN algorithm. We identified an ortholog (GenBank accession no. NM_017986.2, G protein-coupled receptor 172B) that exhibited a high degree of similarity to the rat gene. On the basis of the sequence, a 5'- and a 3'-RACE-PCR were performed with the reverse primers 5'-CTCGGTTGGCTTCTGCAATGGCAAAG-3' and 5'-AACAGCAACAGGAGACCCCAAGGC-3', and the forward primers 5'-CCCTCCATACCTCTCTGTGTTGAGGGC-3' and 5'-CCACCCATCGTATTACGTCG-3' using the human kidney-derived adaptor-ligated Marathon-Ready cDNA library (Clontech). The RACE fragments were ligated and sequenced. We simultaneously obtained an ortholog of rRFT1, hRFT1, and a splice variant hRFT1sv.

**Fig. 1.** A: comparison of the deduced amino acid sequences of human riboflavin transporter 1 (hRFT1), splice variant of hRFT1 (hRFT1sv), and rat RFT1 (rRFT1). The conserved residues in hRFT1 are indicated by dots. The GenBank accession numbers for hRFT1, hRFT1sv, and rRFT1 are AB362533, AB362534, and AB362535, respectively. B: hydropathy plot of hRFT1. C: cDNA sequences of hRFT1 and hRFT1sv. FP1, FP2, FP3, RP1, and RP2 are forward or reverse primers specific for hRFT1 or hRFT1sv, as shown in MATERIALS AND METHODS. ORF is open reading frame of hRFT1. siRNA, small interfering RNA.
These genes were subcloned into the plasmid vector pBK-CMV or pEGFP-C1.

**Cell culture and transfection.** HEK-293 cells (American Type Culture Collection CRL-1573) were cultured in complete medium consisting of Dulbecco’s modified Eagle’s medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in an atmosphere of 5% CO2-95% air at 37°C. The human colon carcinoma cell line Caco-2, obtained from the American Type Culture Collection (ATCC HTB37) was cultured in complete medium consisting of Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum and 1% nonessential amino acids (Invitrogen).

For a transient expression system, pBK-CMV and pEGFP-C1 containing hRFT1, hRFT1sv, and rRFT1 were purified using the Midi-V100TM Ultrapure Plasmid Extraction System (Viogene, Sunnyvale, CA). For the RNA interference system, the sequence of small interfering RNA (siRNA) targeting hRFT1 and hRFT1sv was as follows: 5'-UCCCACCAGCAUCAACCAGUGUU-3' (Fig. 1C). A nonspecific siRNA with the same guanine-cytosine content was used as a control: 5'-AAAAGACCTTCCAGAGGTTG-3' (FP1) and a reverse primer 5'-AGACACCTGTAACCCGAT-3' (RP1) specific for hRFT1, and with a forward primer 5'-ACATGGCATCATTCCTCCT-3' (FP2) and a reverse primer 5'-AGACACCTGTAACCCGAT-3' (RP1) specific for hRFT1sv (Fig. 1C). To examine the effect of siRNA on the sum of the mRNA expression of hRFT1 and hRFT1sv, we used a forward primer 5'-ATTGGGCTGCTGCT-3' (FP2) and a reverse primer 5'-CAGGGGTCTACA-CAGTCCCTT-3' (RP2) specific for hRFT1 and hRFT1sv (Fig. 1C). The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. To determine the sum of the mRNA expression of hRFT1 and hRFT1sv, real-time PCR was carried out as described previously (28).

**Western blot analysis.** Cell lysate was directly prepared from the cells with 1% NP-40, and crude membrane fractions were prepared as described previously, with some modifications (42). Briefly, cells were homogenized by sonication in the buffer (250 mM sucrose and 5 mM HEPES, pH 7.4) and were then centrifuged (2,000 x g, 10 min). The supernatant was recentrifuged (15,000 x g, 10 min), and the pellet was used for the crude membrane samples. Cell lysate fractions (75 µg) and crude membrane fractions (25 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) by semi-dry electrobolting. The blots were probed with specific primer sets. The PCR products of 126 bp and 154 bp correspond to hRFT1 and hRFT1sv, respectively. The quality of the RNA samples was also checked by RT-PCR for GAPDH as an internal control. Plasmid DNAs encoding hRFT1 and hRFT1sv were used as a positive control for hRFT1 and hRFT1sv.

**Fig. 2.** Real-time PCR (A) and the splice variant-specific RT-PCR analysis (B) of hRFT1 and hRFT1sv are shown. **A:** mRNA level of hRFT1 was calculated by the absolute standard method. The primer and probe set for hRFT1 cross-reacted with both hRFT1 and hRFT1sv. Therefore, the values represent the mRNA levels of both hRFT1 and hRFT1sv determined by real-time PCR. The mRNA level of hRFT1 was calculated by the absolute standard method. The primer and probe set for hRFT1 cross-reacted with both hRFT1 and hRFT1sv. Therefore, the values represent the mRNA levels of both hRFT1 and hRFT1sv determined by real-time PCR. The results of the RT-PCR analysis are shown in **B.** The mRNA level of hRFT1 was calculated by the absolute standard method. The primer and probe set for hRFT1 cross-reacted with both hRFT1 and hRFT1sv. Therefore, the values represent the mRNA levels of both hRFT1 and hRFT1sv determined by real-time PCR. The results of the RT-PCR analysis are shown in **B.**

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blocked and incubated overnight at 4°C with a primary antibody specific for green fluorescent protein (GFP; Roche Diagnostics, Indianapolis, IN). The bound antibody was detected on X-ray film using enhanced chemiluminescence with horseradish peroxidase-conjugated secondary antibodies and cyclic diacylhydrazides (GE Healthcare UK, Little Chalfont, United Kingdom).

Fluorescence cytochemistry. HEK-293 cells were transfected with the plasmid vector pEGFP-C1 containing rRFT1, hRFT1, or hRFT1sv as described above. The cells were fixed with 4% paraformaldehyde for 30 min at room temperature and observed using a BX-50-FLA fluorescence microscope (Olympus, Tokyo, Japan). Images were captured with a DP.50 CCD camera (Olympus) using Studio Lite software (Olympus).

Uptake experiment. Cellular uptake of [3H]riboflavin (1.517 TBq/mmol; Moravek Biochemicals, Brea, CA), [N-methyl-3H]cimetidine (451 GBq/mmol; GE Healthcare), [methyl-14C]choline (2.035 GBq/mmol; American Radiolabeled Chemicals, St. Louis, MO), [6,7-3H(N)]estrone-3-sulfate, ammonium salt ([3H]ES; 2.1 TBq/mmol; PerkinElmer Life Analytical Sciences, Boston, MA), and [3H(G)]thiamine (370 GBq/mmol; American Radiolabeled Chemicals) was measured with monolayer cultures grown on poly-D-lysine-coated 24-well plates. The composition of the incubation buffer was as follows: 145 mM NaCl, 3 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 5 mM D-glucose, and 5 mM HEPES (pH was adjusted with NaOH). The Na+-free incubation buffer was prepared by replacing Na+ with N-methyl-D-glucamine or choline. The experimental procedures and the composition of other incubation buffers were described previously (43). Concentration dependence of riboflavin transport was fit by the combination of the Michaelis-Menten equation and a linear relationship:

\[ V = V_{\text{max}} \frac{[S]}{K_m + [S]} + K_d [S], \]

where \( V \) is transport rate, \( V_{\text{max}} \) is the maximal transport rate, \( [S] \) is the concentration of riboflavin, \( K_m \) is the Michaelis-Menten constant, and \( K_d \) is a diffusion constant.

The protein content of the cell monolayers solubilized in 0.5 N NaOH was determined by the method of Bradford with bovine γ-globulin as a standard.

Statistical analysis. Data are expressed as means ± SE. The data were analyzed statistically using an unpaired Student’s t-test. Multiple comparisons were performed using Tukey’s two-tailed test after a one-way analysis of variance. Probability values of less than 0.05 were considered statistically significant.

RESULTS

Isolation and structural analysis of hRFT1, hRFT1sv, and rRFT1. A single clone encoding rRFT1 was isolated and sequenced. The rRFT1 cDNA (GenBank accession no.
AB362535) consists of 2,047 bp with an open reading frame encoding a 450-amino acid protein. It was predicted to have 10 putative membrane-spanning domains by the SOSUI program (10). There is a potential site for NH2-linked glycosylation at position 178 and two consensus sequences for protein kinase COOH-dependent phosphorylation at positions 76 and 278 in rRFT1. Moreover, a human ortholog, hRFT1, and its splice variant, hRFT1sv, were isolated. The hRFT1 cDNA (GenBank accession no. AB362533) consists of 2,305 bp coding for a 448-amino acid protein with 81.1% amino acid identity and 96.4% similarity to rRFT1. It was also predicted to have 10 putative membrane-spanning domains (Fig. 1B). There is a potential site for NH2-linked glycosylation at position 178 and a consensus sequence for protein kinase COOH-dependent phosphorylation at position 438 in hRFT1. The hRFT1sv cDNA (GenBank accession no. AB362534) consists of 2,402 bp coding for a 167-amino acid protein, possessing one putative membrane-spanning domain. The hRFT1 transcript consists of five exons (Fig. 1C). Unlike hRFT1, hRFT1sv was not spliced between exon 2 and exon 3 of hRFT1. Thus, an insertion of 118 bp between exon 2 and exon 3 of hRFT1 was found in hRFT1sv. The amino acid sequences of hRFT1, hRFT1sv, and rRFT1 are shown in Fig. 1A.

Tissue distribution, cellular localization, and functional characterization of hRFT1, hRFT1sv, and rRFT1. The mRNA levels of hRFT1 and hRFT1sv were examined at the mRNA level by real-time PCR as a sum of the expression of both mRNAs (Fig. 2A). The hRFT1 and hRFT1sv mRNAs were expressed strongly in the placenta and small intestine, moderately in the kidney, colon, lung, prostate, uterus, and thymus, and weakly in all other tissues. In addition, we independently detected hRFT1 and hRFT1sv in human tissues by RT-PCR using specific primer sets (Fig. 2B). PCR products of the size expected for hRFT1 and hRFT1sv (147 bp and 155 bp) were found in 21 human tissues; however, in some tissues (hRFT1: heart, skeletal muscle, stomach and liver; hRFT1sv: adrenal gland, prostate, spleen, and uterus), the signals were very weak. To rule out the contamination of genomic DNA, RT-PCR was carried out using the samples that were reverse transcribed with or without Superscript II reverse transcriptase (Supplemental Fig. 1; supplemental material for this article is available online at the American Journal of Physiology-Cell Physiology website). Moreover, the tissue distribution of rRFT1 was determined (Fig. 3). A PCR product of the size expected for rRFT1 was found in 20 rat tissues and especially highly in the adipose, brain, colon, ovary, placenta, small intestine, spleen, and testis.

To visualize the cellular localization of these proteins, HEK-293 cells were transfected with enhanced GFP (EGFP)-tagged hRFT1, hRFT1sv, and rRFT1 (Fig. 4A). Fluorescence was observed in the plasma membrane of the majority of membranes of the cells transfected with EGFP-tagged hRFT1 and rRFT1. A weak signal from EGFP-tagged hRFT1sv was observed in the cytoplasm of only a few cells. In addition, Western blot analysis was performed using the cell lysate and crude membrane of HEK-293 cells transfected with these cDNAs (Fig. 4B). A signal for EGFP-tagged hRFT1 and rRFT1 was strongly detected in the crude membrane and weakly observed in the cell lysate. However, no signal for EGFP-tagged hRFT1sv was observed in the cell lysate and crude membrane.

We screened more than 25 compounds and identified riboflavin as a substrate for RFT1 (Fig. 5 and Supplemental Table). The uptake of [3H]riboflavin was significantly increased by the transfection with hRFT1 cDNA, but not with hRFT1sv cDNA (Fig. 5A). Moreover, uptake was also significantly increased in the rRFT1-transfected cells compared with control cells (Fig. 5B). In addition, uptake of [3H]riboflavin by Xenopus oocytes expressing rRFT1 was also examined (Supplemental Fig. 2). Injection of rRFT1 cRNA into Xenopus oocytes tended to increase the uptake of [3H]riboflavin compared with water-injected oocytes, but this increase was not significant. In the presence of 0.1 mM unlabeled riboflavin, uptake of [3H]riboflavin by Xenopus oocytes was almost completely suppressed.

Uptake of [3H]riboflavin by HEK-293 cells. Before the riboflavin transport activity of the native transporter was evaluated, the mRNA expression of hRFT1 and hRFT1sv was examined. RT-PCR products of a size expected for hRFT1 and hRFT1sv corresponding to the positive controls were found in HEK-293 cells corresponding to positive controls (Fig. 6). Therefore, it is suggested that the [3H]riboflavin transport activity in HEK-293 cells is mediated, at least in part, by hRFT1. The functional characterization of riboflavin transport by HEK-293 cells was carried out. The uptake of [3H]ribofla-
vin in HEK-293 cells increased in a time-dependent manner (Fig. 7A). The replacement of Na\(^+\) with N-methyl-D-glucamine or choline did not affect the \(^3\text{H}\)riboflavin uptake by HEK-293 cells (Fig. 7B). The \(^3\text{H}\)riboflavin uptake was not changed in the presence of high K\(^+\), Ba\(^{2+}\), valinomycin, or high K\(^+\) and valinomycin (Fig. 7C). Moreover, the pH dependence was not observed (Fig. 7D). In the presence of riboflavin analogues, FAD, FMN, and lumiflavin, uptake of \(^3\text{H}\)riboflavin by HEK-293 cells was decreased. On the other hand, probenecid or cimetidine did not influence the \(^3\text{H}\)riboflavin uptake (Fig. 7E). To clarify whether the native \(^3\text{H}\)riboflavin transport activity in HEK-293 cells corresponded to hRFT1, the influence of siRNA specific for hRFT1 and hRFT1sv (hRFT1/hRFT1sv siRNA) was examined. Transfection of control siRNA into HEK-293 cells did not affect the uptake of \(^3\text{H}\)riboflavin compared with nontransfected cells [261.1 ± 4.8 fmol·mg protein\(^{-1}\)·10 min\(^{-1}\) (nontransfected) vs. 256.7 ± 2.6 fmol·mg protein\(^{-1}\)·10 min\(^{-1}\) (control siRNA); \(P = 0.12\)]. The uptake of \(^3\text{H}\)riboflavin was significantly decreased after the transfection with hRFT1/hRFT1sv siRNA (Fig. 8A). RT-PCR analysis confirmed that hRFT1/hRFT1sv siRNA reduced the sum of hRFT1 and hRFT1sv mRNA expression (Fig. 8B). A saturable concentration-dependent uptake of \(^3\text{H}\)riboflavin by HEK-293 cells transfected with hRFT1/hRFT1sv siRNA or control siRNA was observed (Fig. 8C). In addition, the apparent Michaelis-Menten constant (\(K_m\)) and maximal uptake rate...
were compared between the cells transfected with hRFT1/hRFT1sv siRNA and those transfected with the control siRNA. The calculated $K_m$ values were 28.1/2.8 nM and 35.0/4.1 nM ($P = 0.214$), respectively. Transfection with hRFT1/hRFT1sv siRNA significantly decreased the calculated $V_{max}$ value (2,368.3/109.0 vs. vs. 1,662.3/94.2 fmol/mg protein/10 min; $P = 0.003$). We examined the ability of rRFT1 to rescue the knockdown by hRFT1 siRNA (Fig. 8).

Transfection of rRFT1 caused a 1.3-fold and 1.9-fold increase in the riboflavin uptake by control siRNA and hRFT1/hRFT1sv transfected cells, respectively. Moreover, substrate specificity was examined. The uptake of $[^3H]$cimetidine, $[^3H]$estrone sulfate, $[^3H]$choline, and $[^3H]$thiamine was unchanged in HEK-293 cells transfected with hRFT1/hRFT1sv siRNA, although the uptake of $[^3H]$riboflavin was significantly decreased (Fig. 9).

**Uptake of $[^3H]$riboflavin by Caco-2 cells.** Before the activity of the native transporter was evaluated, the mRNA expression of hRFT1 and hRFT1sv in Caco-2 cells was determined (Fig. 6). As shown in Fig. 10A, the uptake of $[^3H]$riboflavin increased in a concentration-dependent manner. The apparent $K_m$ and $V_{max}$ values were 63.7 ± 6.9 nM and 853.4 ± 52.7 fmol/mg protein/10 min$^{-1}$, respectively. To clarify whether the native $[^3H]$riboflavin transport activity in Caco-2 cells corresponds to hRFT1, the influence of hRFT1/hRFT1sv siRNA was examined. The uptake of $[^3H]$riboflavin was significantly decreased after the transfection with hRFT1/hRFT1sv siRNA (Fig. 10B).
DISCUSSION

In the present study, novel human and rat riboflavin transporters (hRFT1 and rRFT1) were successfully identified. The tissue distribution, cellular localization, and functional characterization of hRFT1 suggested that hRFT1 plays an important role in the cellular uptake of riboflavin. An inactive splice variant of hRFT1, hRFT1sv, was also cloned. The hRFT1sv cDNA contains a 118-bp insertion between exon 2 and exon 3 of hRFT1. Transfection of hRFT1sv in HEK-293 cells which natively express hRFT1 did not affect the riboflavin uptake (Fig. 5A). In addition, EGFP-tagged hRFT1sv was observed in the cytoplasm of only a few cells and was not detected by Western blot analysis (Fig. 4). These results suggested that hRFT1sv is not a membrane protein and that hRFT1sv is neither an active transporter nor a dominant negative. The physiological role of hRFT1sv remains unclear and should be examined in future studies.

Mammals are unable to synthesize riboflavin and thus must acquire it from exogenous sources. The absorption of riboflavin from foods takes place predominantly in the small intestine through an active carrier-mediated transport process (17, 24, 41). In addition, carrier-mediated absorption in the colon is thought to be important, because riboflavin is synthesized by bacterial metabolism in the colon (21, 44). The high levels of hRFT1 in the small intestine and colon suggest that hRFT1 mediates the absorption of riboflavin at these sites (Fig. 2). The apparent $K_m$ value of riboflavin uptake by human intestinal brush-border membrane vesicles was 7.3 $\mu$M (34) and that by rat intestinal basolateral membrane vesicles was 5.0 $\mu$M (35). Previous studies have demonstrated apparent $K_m$ values of 7.3 $\mu$M for riboflavin uptake by human intestinal brush-border membrane vesicles (34), and 5.0 $\mu$M for uptake by rat intestinal basolateral membrane vesicles (35). Corresponding $K_m$ values for uptake by intestinal cells are 63.7 $\mu$M in Caco-2 cells (the present study; Fig. 10A), 0.30 $\mu$M in Caco-2 cells (36), and 0.14 $\mu$M in NCM460 cells (37). The $K_m$ values associated with intestinal membrane vesicles are higher than those of intestinal cell lines. In the present study, the uptake of riboflavin by Caco-2 cells was partially inhibited by hRFT1/hRFT1sv siRNA (Fig. 10B). The binding constant ($K_b$) for the rat intestinal brush-border membrane has been calculated to be 0.07 $\mu$M and 0.12 $\mu$M, suggesting that more than two binding sites exist in the brush-border membrane (2). In addition, the uptake of riboflavin by HEK-293 cells, whose riboflavin uptake was partially mediated by hRFT1 (Fig. 8A), was independent of Na$^+$, membrane potential, or pH (Fig. 7, B–D) and was completely inhibited by riboflavin analogues (Fig. 7E) as previously reported and discussed (36). These results and previous reports suggested that hRFT1 could play, at least in part, a role in the absorption of riboflavin in the intestine as a high affinity transporter.

We previously constructed a rat kidney mRNA expression database by sequencing cDNA clones randomly selected from a rat kidney cDNA library (12). This database included 2,048 genes, most of which are abundantly expressed and physiologically significant in the kidney. There are 61 transporters (3.7% of the 1,666 known-function genes) including organic ion transporters (OAT1, OAT3, and OCTN2), Na$^+$-coupled phosphate cotransporter II (NaPi-IIa and NaPi-IIc), and aquaporin 2 (AQP2). These transporters have important physiological roles

Table 1. hRFT1 homologs registered in the GenBank database: identity and similarity to hRFT1

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hRFT1, human riboflavin transporter 1; rRFT1, rat riboflavin transporter 1.
in the kidney (8, 16, 29, 30). A recent update of this database revealed the characteristics of some unknown genes. We focused on transmembrane proteins and successfully identified NaGLT1, which mediates the tubular reabsorption of glucose and fructose (11, 13). In addition, this database includes a Slc5a8, which was characterized as a monocarboxylate transporter, and is thought to be a tumor suppressor (27, 31). Moreover, mouse Oatp3 was also identified (3). Therefore, this database represents a useful resource for identifying the physiologically important transporters in the kidney.

We have twice attempted to raise an antibody against hRFT1 but have not succeeded in obtaining a functional one. The same amino acid sequence of hRFT1 has already been registered as GPR172B (accession no. NP_001980.47) in the GenBank database, although its substrates and physiological function have yet to be determined. In addition, this gene was previously reported to be the human ortholog of the porcine receptor for endogenous retrovirus A, which mediates the infection of the cells with porcine endogenous retrovirus (6). Because its amino acid sequence is similar to that of a G protein-coupled receptor family member and a multimembrane spanning protein, it is anticipated that it will be difficult to produce a functional antibody against hRFT1, as previously reported (9, 22, 39). In the present study, EGFP-tagged hRFT1 was expressed in the plasma membrane (Fig. 4), suggesting that hRFT1 mediates the transport of riboflavin across the plasma membrane. An antibody against hRFT1 will, however, reveal its detailed distribution in the tissues.

The RibU protein of Lactobacillus lactis was identified to be a bacterial riboflavin transporter containing five putative transmembrane domains (1, 5). Its homologues were identified among bacterial genes but not among mammalian genes (26). The Mch5p protein of Saccharomyces cerevisiae is an ortholog of the mammalian monocarboxylate transporter and has been reported to transport riboflavin; the transport of riboflavin by the mammalian monocarboxylate transporter has, however, not been assessed (33). hRFT1 and rRFT1 exhibit no significant similarity to RibU, Mch5p, or SLC families; therefore, mammalian riboflavin transporters (hRFT1 and rRFT1) appear to belong to a novel mammalian riboflavin transporter family and not to the bacterial riboflavin transporter family. A BLAST search of the GenBank database identified homologues of hRFT1 from the following mammalian, fish, and amphibian species: Pan troglodytes, Macaca mulatta, Papio hamadryas, Canis lupus, Sus scrofa, Bos taurus, Equus caballus, Mus musculus, Monodelphis domestica, Xenopus laevis, and Danio rerio. The mammalian genes exhibit high levels of identity (>69%) and similarity (>91%) to hRFT1 (Table 1). On the basis of these results, it is suggested that these genes should be classified into a novel mammalian riboflavin transporter family.

In conclusion, hRFT1 and rRFT1 were identified and characterized as novel mammalian riboflavin transporters using our rat kidney mRNA expression database (12). In addition, an inactive splice variant, hRFT1sv, was cloned. RFT1 could play an important role in the homeostasis of riboflavin.

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