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Identification and Functional Characterization of Rat Riboflavin Transporter 2

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We have newly identified rat riboflavin transporter 2 (rRFT2) and its human orthologue (hRFT2), and carried out detailed functional characterization of rRFT2. The mRNA of rRFT2 was highly expressed in jejunum and ileum. When transiently expressed in human embryonic kidney (HEK) 293 cells, rRFT2 could transport riboflavin efficiently. Riboflavin transport mediated by rRFT2 was Na^+-independent but moderately pH-sensitive, being more efficient in acidic conditions than in neutral and basic conditions. Kinetic analysis indicated that rRFT2-mediated riboflavin transport was saturable with a Michaelis constant (K_m) of 0.21 μM. Furthermore, it was specifically and strongly inhibited by lumiflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and to a lesser extent by amiloride. Such ability to transport riboflavin in a specific manner, together with its high expression in the small intestine, indicates that RFT2 may play a role in the intestinal absorption of riboflavin.

Key words: absorption, intestine, riboflavin, RFT2, transporter.

Abbreviations: BSP, sulfobromophthalein; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; RFT, riboflavin transporter; RT, reverse transcription; SDS, sodium dodecyl sulphate; TRIS, tris(hydroxymethyl)aminomethane.

Riboflavin (vitamin B_2) is enzymatically converted in the body to flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which function as cofactors for a number of redox enzymes and play important roles in the regulation of cellular physiology and bioenergization utilizing glucose, fatty acids, glycerol and amino acids (*1*, *2*). Since human and other mammals cannot synthesize riboflavin *de novo*, its supply to the body absolutely depends on the exogenous sources of diet and, in part, products by intestinal microflora (*3*). To secure such riboflavin supply, a specialized transport mechanism would be needed in the small intestine for its efficient absorption, since this vitamin would be otherwise absorbed only poorly by passive diffusion across intestinal epithelial cells due to its water soluble nature. In fact, the existence of a carrier-mediated transport system specific for riboflavin has been suggested by studies using intestinal tissue specimens, brush border membrane vesicles and cell lines of rat and/or human (*4–12*). However, the identification of its molecular entity has not been accomplished yet, imposing difficulties in further elucidation of the mechanisms of riboflavin absorption and its regulation. Although riboflavin transporter 1 (RFT1) was reported to be identified during the course of our present study, its role in riboflavin absorption has not been fully clarified yet (*13*).

We here report on our newly identified riboflavin transporter, which may be involved in the intestinal absorption of riboflavin. Since our identified transporter was found to be homologous to (RFT1), it is herein referred to as RFT2. We focused on its rat orthologue (rRFT2) to examine its tissue distribution in detail together with its functional characteristics. We could successfully accomplish the functional characterization of cloned rRFT2 and found its high expression in the small intestine. The finding of RFT2 is a significant and important step forward toward elucidation of the mechanisms of intestinal riboflavin absorption and its regulation.

MATERIALS AND METHODS

Materials—[^{3}H]Riboflavin (24 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA). Riboflavin was obtained from Wako Pure Chemicals (Osaka, Japan), and FAD, FMN and lumiflavin were from Sigma-Aldrich (St Louis, MO, USA). Culture medium and fetal calf serum (FCS) were obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade and commercially obtained.

Cell Culture—Human embryonic kidney (HEK) 293 cells were maintained at 37°C and 5% CO_2 in Dulbecco's

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modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Isolation of rRFT2—Total RNA was prepared from the rat small intestine by a guanidine isothiocyanate extraction method (*14*). A reverse transcription (RT) reaction was carried out to obtain cDNA mixture, using 3 μg of the total RNA, an oligo(dT) primer, and ReverTra Ace (Toyobo, Osaka, Japan) as a reverse transcriptase. The cDNA of rRFT2 was isolated from thus obtained cDNA mixture by polymerase chain reaction (PCR) amplification of cDNA containing the open reading frame of rRFT2, using KOD plus DNA polymerase (Toyobo) and the following primers: forward primer, 5′-ACA ATG GCC TTC CTG ACA CA-3′; reverse primer, 5′-AGT AGG GCA AAG GGT TGT TAC-3′. These primers were designed based on the sequence in GenBank (accession no. NP_001032275.1). PCR was performed using the following conditions: 94°C for 1 min; 33 cycles of (i) 94°C for 20 s, (ii) 56°C for 20 s and (iii) 68°C for 1.5 min. The second PCR was performed using the PCR product as a template and a forward primer containing an *Eco*RI restriction site (underlined), 5′-TTG AAT TCC ACA ATG GCC TTC CTG ACA-3′, and a reverse primer containing an *Xba*I restriction site (underlined), 5′-AAT CTA GAT TCA GCA ATG GGG GTA C-3′. Then the amplified product was introduced at the *Eco*RI and *Xba*I sites into a mammalian expression vector, pCI-neo (Promega, Madison, WI, USA). The sequence of the final product was determined with an automated sequencer (ABI PRISM 3100; Applied Biosystems, Foster City, CA, USA).

The cDNA of hRFT2 (GenBank accession no, NP_212134.3) was isolated similarly by RT-PCR cloning from the human small intestine total RNA (Clontech, Mountain View, CA, USA). The primers for the initial PCR were as follows: forward primer, 5′-CAA AAA GGT GCA TAT ACC AC-3′; reverse primer, 5′-TGC GTT CAT GAT TCA ATT AC-3′. The primers for the second PCR were as follows: forward primer containing an *Eco*RI restriction site (underlined), 5′-TTG AAT TCC ACC GCC ATG GCC TTC CTG ATG-3′; reverse primer containing a *Sal*I restriction site (underlined), 5′-TCA GTC GAC TGC GTT CAT GAT TCA ATT AC-3′.

The cDNAs of rat RFT1 (rRFT1) and human RFT1 (hRFT1) were also isolated similarly using primers designed based on the sequences in GenBank (accession nos, NP_001103140.1 and NP_001098047.1, respectively). The primers for the initial PCR were as follows: forward primer for rRFT1, 5′-GGT GAA AGA AGC TTG ACC CTT-3′; reverse primer for rRFT1, 5′-TGT TTT ATT GTG CAA ATC CCA-3′; forward primer for hRFT1, 5′-GTC GCT GTA CCC AAA CGC ACA-3′; reverse primer for hRFT1, 5′-GGT GGG GTG GAG TTG GGT C-3′. The primers for the second PCR were as follows: forward primer containing an *Eco*RI restriction site (underlined) for rRFT1, 5′-AAG AAT TCC GTA ATG GCA GCA CCT CCA CTG-3′; reverse primer containing an *Xba*I restriction site (underlined) for rRFT1, 5′-TTT CTA GAC AAG TGT GAA GTA ACT CC-3′; forward primer containing an *Eco*RI restriction site (underlined) for hRFT1, 5′-GAG AAT TCC GGA ATG GCA GCA CCC AC-3′; reverse primer containing an *Xba*I restriction site (underlined) for hRFT1, 5′-GGT CTA GAG GTG GGG TGG AGT TGG G-3′.

Generation of GFP-Tagged rRFTs—To generate rRFT2 fused with green fluorescent protein (GFP-rRFT2), the cDNA fragments containing the coding sequence of rRFT2 were prepared by digestion of the pCI-neo-based plasmids carrying rRFT2 cDNA with *Eco*RI and *Xba*I, and then introduced into pEGFP-C1 vector (Clontech). The pCI-neo-based plasmids carrying rRFT1 cDNA were similarly processed for the generation of GFP-rRFT1.

Northern Blot Analysis—The tissue distribution of rRFT2 transcripts in the rat was examined by Northern blots of total RNA samples. Total RNA isolated by guanidine isothiocyanate extraction from various rat tissues (25 μg each) was separated on 1% formaldehyde-agarose gels and transferred to a Hybond-XL nylon membrane (GE Healthcare Bio-sciences, Piscataway, NJ, USA). The UV cross-linked membrane was then hybridized at high stringency with rRFT2-specific cDNA probe, which was labelled with [α-^{32}P]dCTP by use of a random primer DNA labeling kit (GE Healthcare Bio-sciences). The 18S ribosomal RNA (rRNA) was stained with ethidium bromide and used as a reference.

RT–PCR Analysis—Total RNA isolated from various rat tissues (3 μg each) was used for cDNA synthesis, as in the isolation of rRFT2. PCR reactions for rRFT2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference were performed using Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) and the following primers: forward primer for rRFT2, 5′-ATG AGC CAG CTG CCC ACG TA-3′; reverse primer for rRFT2, 5′-ACC ATT GGT GAG CGC ATT GA-3′; forward primer for GAPDH, 5′-CCA TCA CCA TCT TCC AGG AG-3′ reverse primer for GAPDH, 5′-CCT GCT TCA CCA CCT TCT TG-3′. PCR conditions were as follows: 94°C for 2 min; 35 cycles (rRFT2) or 30 cycles (GAPDH) of (i) 94°C for 20 s, (ii) 60°C for 20 s and (iii) 72°C for 30 s. The predicted size of the RT–PCR product was 543 bp for rRFT2.

Transport Study in HEK293 Cells Transiently Expressing Cloned Transporters—HEK 293 cells (1.5×10^5 cells/well initially) were grown on 24-well plates coated with poly-L-lysine for 12 h, transfected with 1 μg/well of the plasmid carrying rRFT2 cDNA by using 3 μl/well of HilyMax (Dojindo Laboratories, Kumamoto, Japan) as a transfection reagent, according to the manufacturer's instructions, and cultured for 36 h for transient expression. Cells in each well were preincubated for 5 min in 1.5 ml of substrate-free uptake buffer containing 140 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.0 mM $CaCl_2$, 25 mM glucose, and 10 mM MES (pH 6.0 and below) or HEPES (pH 6.5 and above), and then transport assays were started by replacing the substrate-free uptake buffer with one containing [^{3}H]riboflavin (0.25 ml). All the procedures were conducted at 37°C. Assays were stopped by addition of ice-cold substrate-free uptake buffer (2 ml), and the cells were washed two times with 2 ml of the same buffer. The cells were solubilized in 0.5 ml of 0.2 M NaOH solution containing 0.5% SDS at room temperature for 1 h, and the associated radioactivity was determined by liquid scintillation counting, using 3 ml of Clear-sol I (Nacalai Tesque, Inc., Kyoto, Japan) as a scintillation fluid, for the evaluation of uptake. Cellular protein

content was determined by the method of Lowry *et al*., using bovine serum albumin as the standard (*15*). The specific uptake of riboflavin by rRFT2 was estimated by subtracting the uptake in mock cells, which were transfected with empty pCI-neo vector, from that in rRFT2-transfected cells.

Similarly, the other transporters were transfected into HEK293 cells for transient expression and transport assays were conducted.

Western Blot Analysis—HEK 293 cells transiently expressing GFP-rRFT2 or GFP-rRFT1 and cultured on the 24-well plates were washed twice with ice-cold phosphate-buffered saline (pH 7.4), scraped off, and pelleted by centrifugation at $800 \times g$ for 3 min at 4°C. The cell pellet was homogenated by sonication in an ice-cold buffer (pH 7.4) containing 250 mM sucrose, 150 mM NaCl, 20 mM Tris–HCl and supplemented with, according to the manufacturer's instructions, protease inhibitor cocktail (Sigma-Aldrich), and then centrifuged at $2{,}000 \times g$ for 10 min at 4°C. The supernatant was recentrifuged at $15{,}000 \times g$ for 30 min at 4°C, and the resultant pellet was used as the crude membrane fraction sample. The sample (30 μg) was separated on the 10% SDS–polyacrylamide gel by electrophoresis and transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (pH 7.4)/0.1% Tween 20 and then probed with primary antibody/mouse anti-GFP (JL-8; Clontech) at a dilution of 1:2,000 for 1 h at room temperature. After washing three times with Tris-buffered saline (pH 7.4)/Tween 20, the membrane was incubated with secondary antibody/goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich) at a dilution of 1:10,000 for 1 h at room temperature. Then, the expression levels of GFP-rRFT2 and GFP-rRFT1 were determined by enhanced chemiluminescence using ECL reagent (Millipore, Billerica, MA, USA), according to the manufacturer's instructions.

Fluorescent Microscopy—HEK 293 cells transiently expressing GFP-rRFT2 or GFP-rRFT1 and cultured on 24-well plates were detached using 0.05% EDTA in phosphate-buffered saline (pH 7.4), seeded at a density of 0.5×10^5 cells on a 35-mm glass bottom dish coated with poly-L-lysine, and cultured overnight. The fluorescent images of GFP-rRFT2 and GFP-rRFT1 were directly visualized by using a confocal laser-scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

Data Analysis—The saturable transport of riboflavin was analysed by assuming Michaelis–Menten type carrier-mediated transport represented by the following equation: $v = V_{max} \times s/(K_m + s)$. The maximum transport rate (V_{max}) and the Michaelis constant (K_m) were estimated by fitting this equation to the experimental profile of the uptake rate (v) versus the substrate (riboflavin) concentration (s), using a non-linear least-squares regression analysis program, WinNonlin (Pharsight, Mountain View, CA, USA), and the reciprocal of variance as the weight. The v in the presence of an inhibitor can be expressed as follows: $v = v_0/(1 + i/IC_{50})$. The half-inhibition concentration (IC_{50}) was estimated together with v in the absence of inhibitors (v_0) by fitting this equation to the experimental profile of v *versus* the inhibitor concentration (i). The parameters are presented as the computer-fitted ones with SE.

Experimental data are represented as the means ± SE, and statistical analysis was performed by using analysis of variance (ANOVA) followed by Dunnett's test, with $P < 0.05$ considered significant.

RESULTS AND DISCUSSION

Identification of rRFT2—In order to first identify a transporter that is involved in the absorption of riboflavin in the rat small intestine, BLAST (Basic Local Alignment Search Tool) searches of the NCBI (National Center for Biotechnology Information) databases were performed using the amino-acid sequence of impX, of which the gene is located upstream of riboflavin operon on the genome of *Fusobacterium nucleatum* and predicted to encode a new type of bacterial riboflavin transporter (*16*), as query. The search results were bioinformatically analysed to find genes that encode putative multi-transmembrane proteins with unknown function. For 20 of them, we could successfully isolate cDNAs from the rat small intestine by RT–PCR cloning. Among them, the cDNA of rRFT2, which is thus referred to as it was found to be homologous to recently identified rRFT1 with 57% similarity (Fig. 1A), is the only one that could enhance riboflavin uptake significantly, compared with that in mock cells, when introduced into HEK293 cells (data shown below in the section of functional characteristics). It was further found that the cDNA of rRFT2 is identical with that of hypothetical protein LOC311536 in GenBank. We also found a cDNA that encodes an orthologous protein in human (hypothetical protein LOC113278), which is herein referred to as hRFT2. As shown in Fig. 1, rRFT2 consists of 463 amino acids and has 11 potential membrane spanning domains with a large extracellular loop between the fifth and sixth transmembrane domains, in which a putative *N*-glycosylation site (Asn^{168}) is located. hRFT2 consists of 469 amino acids with 83% similarity to rRFT2.

It should also be noted that recently identified rRFT1 and its 87% similar orthologue in human (hRFT1) had originally been identified as rat G protein-coupled receptor 172B (rGPR172B) and its human orthologue (hGPR172B). Although they had been suggested to belong to a group of porcine endogenous retrovirus A receptors and mediate porcine retroviral infection, their molecular functions had been left unclear.

Tissue Distribution of rRFT2 mRNA—Northern blot analysis revealed that rRFT2 mRNA (~2.5 kb) is expressed highly in jejunum and ileum and also in testis, and to lesser extents in lung, kidney, stomach and colon, as shown in Fig. 2. Although RT–PCR analysis revealed its expression in all the tissues examined, it is evident that small intestine is the primary site of expression for rRFT2, indicating a possibility that it may be involved in riboflavin absorption. The role of rRFT2 in other tissues with relatively high expression, such as testis, should also be of interest, although information about the disposition characteristics of riboflavin in such tissues is scarce.

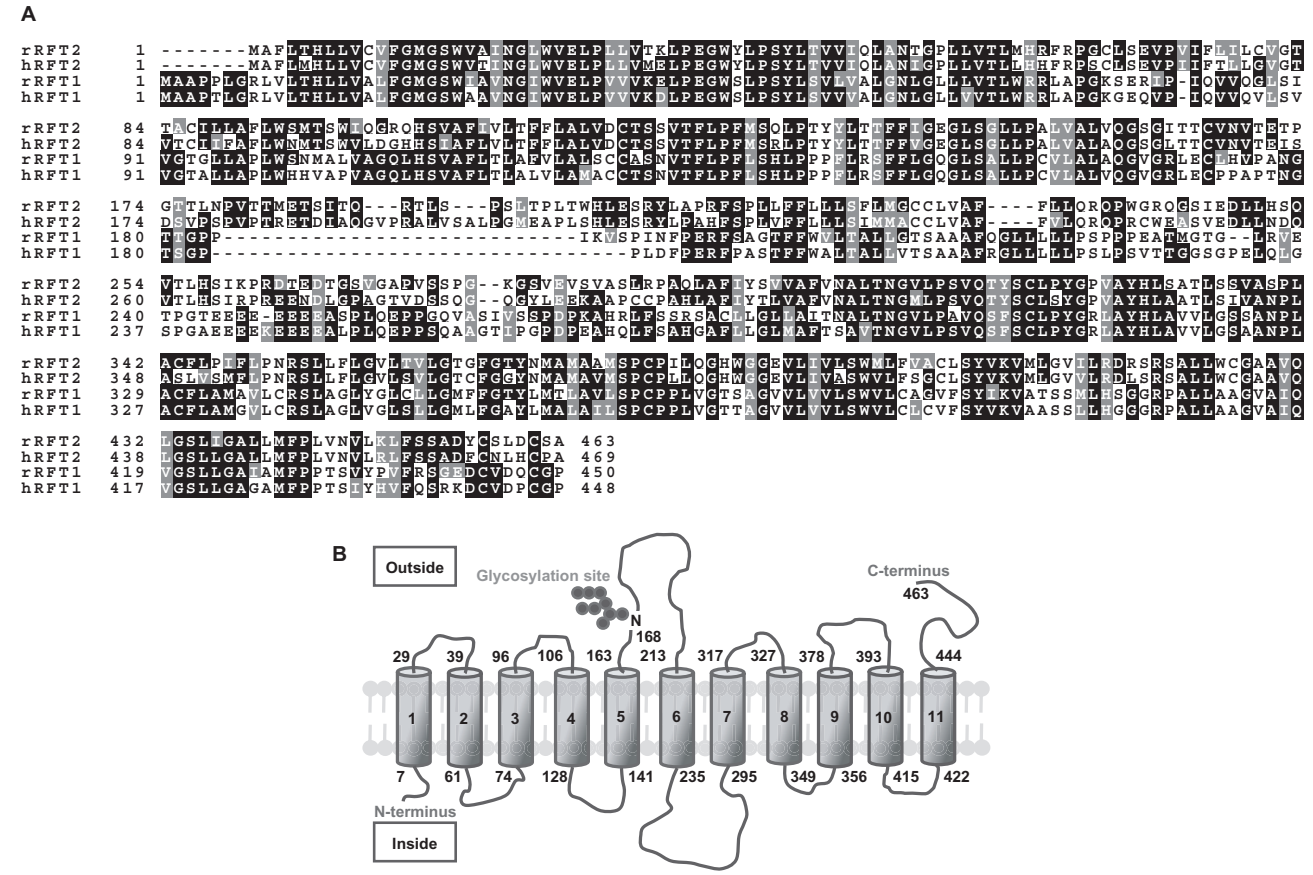

Fig. 1. **Predicted structure of rRFT2.** (A) Aligned amino-acid sequence of rRFT2 with those of hRFT2, rRFT1 and hRFT1. Black and gray boxes highlight sequences of identity and similarity, respectively. (B) Hypothetical membrane topology of rRFT2 by hydropathy analysis using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and by analysis using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) for potential N-glycosylation sites.

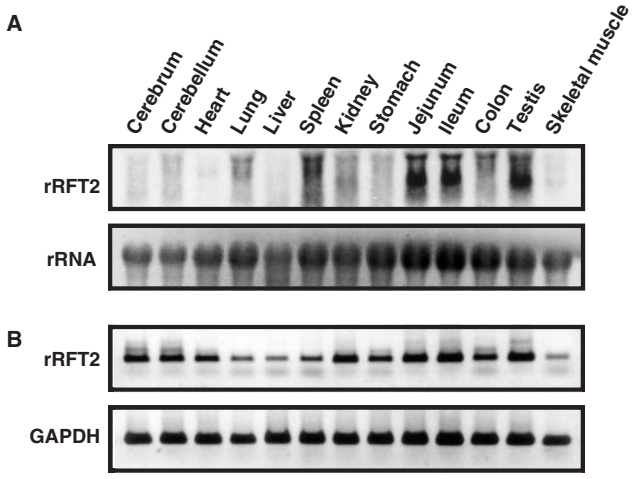

Fig. 2. **Northern blot (A) and RT-PCR (B) analyses for rRFT2 distribution.** The bands of ethidium bromide-stained 18S ribosomal RNA (rRNA) and GAPDH are also shown as references.

Functional Characteristics of rRFT2—rRFT2 was introduced into HEK293 cells by transient expression and the initial examination of its riboflavin transport activity was conducted at pH 7.4 and also pH 6.0, a weakly acidic pH expected in the microclimate at the intestinal surface (Fig. 3A). It was found that rRFT2 can transport riboflavin efficiently, as indicated by about 5-fold greater uptake of riboflavin (5 nM) in rRFT2-expressing cells than in mock cells at pH 6.0 and about 4-fold greater uptake at pH 7.4. hRFT2 was found to be able to transport riboflavin similarly. In contrast, rRFT1 and hRFT1 could transport riboflavin only slightly. Riboflavin transport activities of these transporters observed in the recent study were also only modest with increases in the uptake of riboflavin (5 nM) by about 70% in HEK293 cells transiently expressing them, compared with that in mock cells (*13*). Thus, apparently, riboflavin transport activities of rRFT1 and hRFT1 seem to be much lower, if any, than those of rRFT2 and hRFT2. Transporting riboflavin may not be the primary function for RFT1.

To examine the expression of rRFTs at the plasma membrane, we used GFP-tagged rRFTs. As shown in Fig. 4, the expression of GFP-rRFT2 at the plasma membrane of HEK293 cells was found to be comparable with that of GFP-rRFT1 by fluorescent microscopic observation of the cells (panel A) and western blot of the plasma membrane fractions (panel B). However, GFP-rRFT2-transfected cells showed a much greater riboflavin

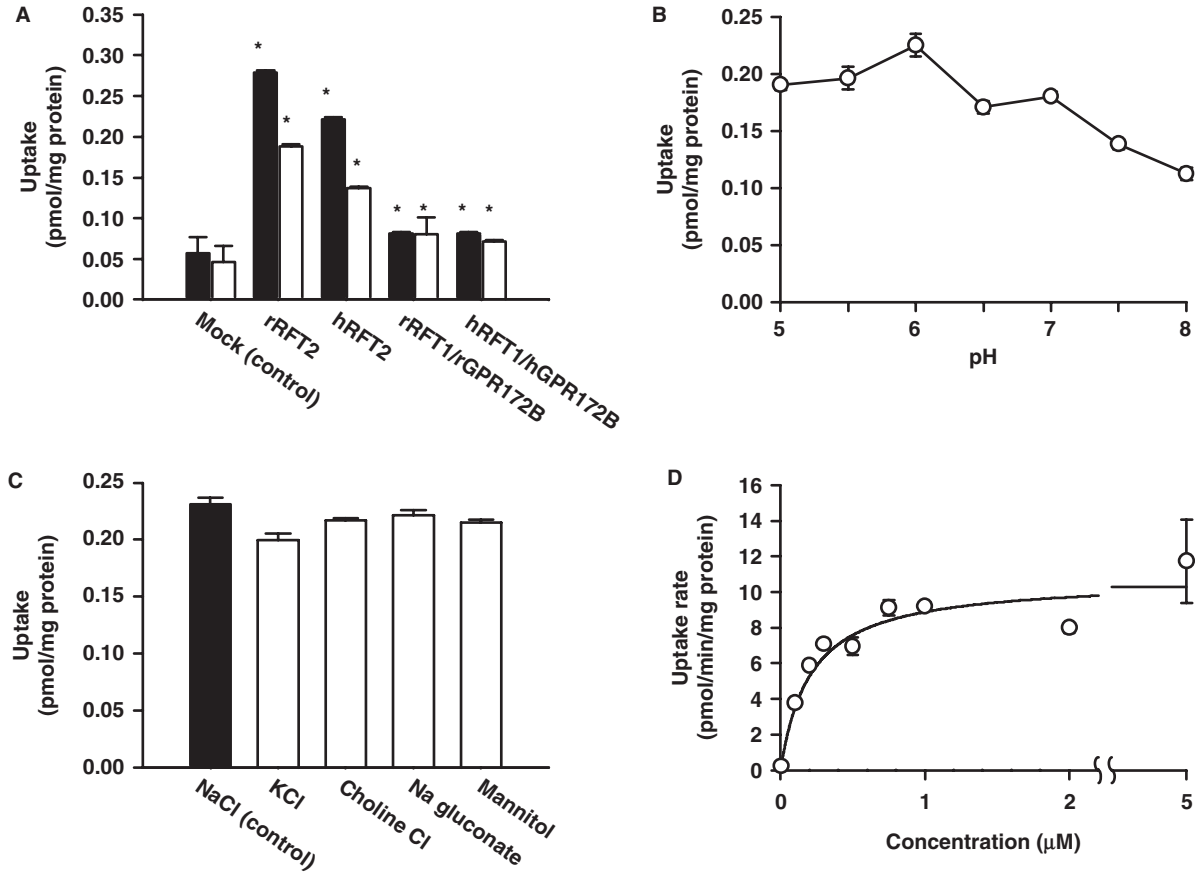

Fig. 3. **Functional characteristics of rRFT2 transiently expressed in HEK293 cells.** (A) Uptake of [^{3}H]riboflavin (5 nM) at pH 6.0 (filled bars) and pH 7.4 (open bars) in HEK293 cells transiently expressing rRFT2, hRFT2, rRFT1 and hRFT1, and in mock cells. (B) Effect of extracellular pH on the rRFT2-specific uptake of [^{3}H]riboflavin (5 nM). (C) Effect of extracellular ions on the rRFT2-specific uptake of [^{3}H]riboflavin (5 nM). NaCl in the control medium was replaced as indicated. (D) Concentration dependence of the rRFT2-specific uptake rate of [^{3}H]riboflavin. The V_{max} and K_m are 11 ± 1 pmol/min/mg protein and 0.21 ± 0.03 μM, respectively, as the computer-fitted parameters with SE. In all experiments, uptake measurements were conducted for 1-min period at 37°C and, unless otherwise indicated, at pH 6.0. Data are represented as the means ± SE ($n = 4$). $^*P < 0.05$ compared with the value for each mock control.

transport activity than GFP-rRFT1-transfected ones (Fig. 4C), consistent with the results for untagged rRFTs (Fig. 3A). These results further indicate that rRFT2 would intrinsically have a much greater riboflavin transport activity than rRFT1.

The specific uptake of riboflavin (5 nM) by rRFT2 was greatest in the acidic pH range of 5.0–6.0 and, above that, decreased with an increase in pH, reaching an approximately 50% lower level at pH 8.0 (Fig. 3B). Thus, rRFT2-mediated riboflavin transport was found to be sensitive to pH, although it does not seem to be entirely dependent on or coupled with H^+. rRFT2-mediated riboflavin transport was found not to depend on Na^+ either, as indicated by no effect of the replacement of NaCl in the medium with KCl, choline Cl or mannitol on the rRFT2-specific riboflavin uptake (Fig. 3C). No effect of the replacement with mannitol indicates that Cl^- is not involved, either. It is supported by the result that the replacement with Na gluconate caused no effect. In addition, the result for the replacement with KCl, which depolarizes the plasma membrane by a high concentration of extracellular K^+, suggests that rRFT2-mediated riboflavin transport is not influenced by membrane potential. From all these results, it is likely that rRFT2-mediated riboflavin transport is of electroneutral facilitated diffusion type, although the mechanism of the pH sensitivity is unclear.

For further transport assessments, we decided to use pH 6.0 as a pH that makes rRFT2 operate most efficiently and would be expected in the microclimate at the intestinal surface. The uptake period was set to be 1 min within the initial phase where uptake was in proportion to time (data not shown).

Kinetic analysis indicated that rRFT2-mediated riboflavin transport was saturable with a V_{max} of 11 pmol/min/mg protein and a K_m of 0.21 μM (Fig. 3D). The K_m, which indicates a quite high affinity of rRFT2 for riboflavin, is comparable with those reported for the Na^+-independent type of carrier-mediated riboflavin transport systems in human-derived cell lines, 0.30 μM in Caco-2 (small intestinal model) (*11*) and 0.14 μM in NCM460 (colonic model) (*12*), and also those reported for the Na^+-dependent type in the rat small intestine, 0.54 μM in isolated and perfused intestine (*5*), 0.15 μM (*6*)

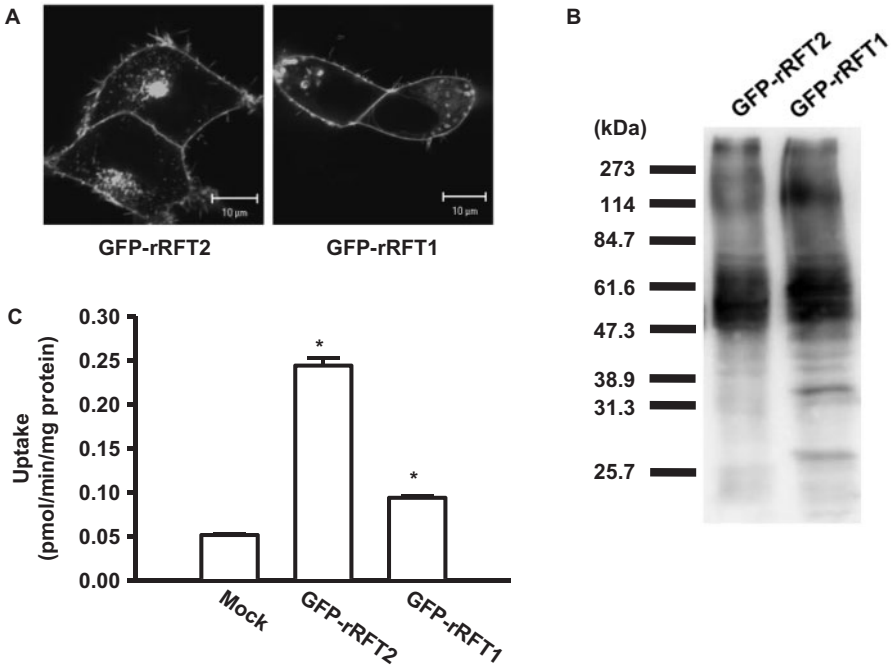

Fig. 4. **Expression of GFP-rRFTs at the plasma membrane of HEK293 cells by transient transfection.** (A) Fluorescent images that show GFP-rRFTs outlining the cells at the plasma membrane in white. (B) Western blots of the plasma membrane fractions (30 μg protein aliquots), which were probed for tagged GFP and show the bands of GFP-rRFT2 and GFP-rRFT1 at about 60 kDa. (C) Uptake of [^{3}H]riboflavin (5 nM) for 1 min at 37°C and pH 6.0 in HEK293 cells transiently expressing GFP-rRFT2 and GFP-rRFT1, and in mock cells. Data are represented as the means ± SE ($n = 4$). $^*P < 0.05$ compared with the value for mock control.

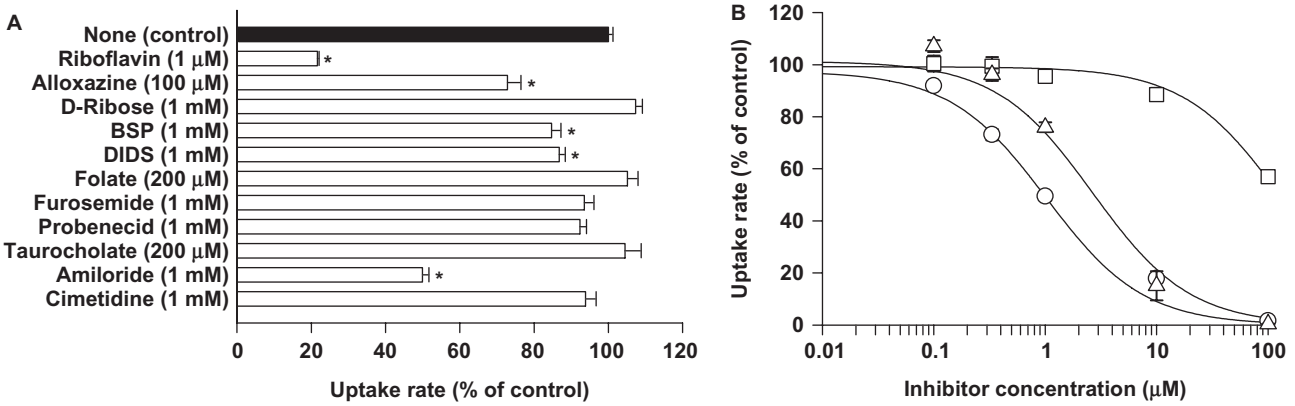

Fig. 5. **Substrate specificity of rRFT2 transiently expressed in HEK293 cells.** (A) Effect of various compounds on the rRFT2-specific uptake rate of [^{3}H]riboflavin (5 nM). (B) Effect of varied concentrations of riboflavin derivatives (inhibitors), lumiflavin (open circle), FMN (open triangle) and FAD (open square), on the rRFT2-specific uptake rate of [^{3}H]riboflavin (5 nM). The IC_{50} values for lumiflavin, FMN and FAD are 1.02 ± 0.18, 2.45 ± 0.28 and 129 ± 10 μM, respectively, as the computer-fitted parameters with SE. In all experiments, uptake measurements were conducted for 1-min period at 37°C and pH 6.0, and test compounds were added only to the buffer for uptake period. The control values for normalization were 0.222 ± 0.006 and 0.219 ± 0.004 pmol/min/mg protein, respectively, in panels A and B. Data are represented as the means ± SE ($n = 4$). $^*P < 0.05$ compared with the value for control.

and 0.57 μM (*7*) in everted tissue sacs, and 0.25 μM (*8*) and 0.12 μM (*9*) in brush border membrane vesicles.

Substrate Specificity of rRFT2—To help clarify the substrate specificity of rRFT2, we examined the effect of various compounds on the rRFT2-specific uptake of riboflavin (5 nM). As shown in Fig. 5A, D-ribose, which forms the side-chain attached to the alloxazine ring in riboflavin molecule, was found not to exhibit significant inhibitory activity at 1 mM, whereas alloxazine did at 100 μM, though reducing the rRFT2-specific uptake only modestly by about 30%. Organic anions such as sulfobromophthalein (BSP), 4,4′-diisothiocyanostilbene-2, 2′-disulfonate (DIDS), folate, furosemide, probenecid and taurocholate, and cimetidine, an organic cation, exhibited

no or only minimal inhibitory activity at their concentration as high as 200 μM or 1 mM. Only amiloride, an organic cation, was found to exhibit significant inhibitory activity at 1 mM, reducing the rRFT2-specific uptake by about 50%.

On the other hand, riboflavin derivatives such as lumiflavin, FMN and FAD exhibited significant inhibitory activity at lower concentrations of 100 μM and below (Fig. 5B). It was suggested that lumiflavin has the highest affinity for rRFT2 (IC_{50} of 1.02 μM), FMN has the second highest affinity (2.45 μM) and FAD has a much lower affinity (129 μM). It is notable, however, that riboflavin has about 5-fold higher affinity (K_m of 0.21 μM) than lumiflavin, suggesting that it is the most preferred substrate for rRFT2 among them. All these results indicate that rRFT2 is highly specific to riboflavin and some derivatives, implying its nature as a riboflavin transporter.

Physiological Role of RFT2—The functional characteristics of rRFT2 are particularly in agreement with those reported for the carrier-mediated riboflavin transport system in the Caco-2 cell line as a small intestinal model (*11*). rRFT2 and the transport system in Caco-2 cells are both Na^+-independent but moderately pH-sensitive, being more efficient in acidic conditions than in neutral and basic conditions, and have comparative affinities (K_m values) for riboflavin. They also have similar characteristics with regard to recognition of substrates (or inhibitors), typically indicated by strong inhibition by lumiflavin, no inhibition by D-ribose, and weak but significant inhibition by amiloride. We probably can expect similar characteristics for hRFT2 as it is highly (83%) similar to rRFT2, though we have not completed its detailed characterization yet. Riboflavin transport systems reported in rat and human intestines also have similar characteristics with regard to affinity for riboflavin and recognition of substrates (or inhibitors), but they are known to be Na^+-dependent (*5–10*). Thus, although the issue of Na^+ requirement remains inconclusive, it is at least likely that RFT2 is the molecular entity of the transport system characterized in the Caco-2 cell line and may play a role in the intestinal absorption of riboflavin. There remains, however, a possibility that Na^+-dependent transport by an as yet unidentified transporter might be responsible for the brush border uptake of riboflavin in the small intestine, and RFT2 might be involved in the basolateral efflux as a facilitative transporter, analogously to a typical absorption process of nutrients like D-glucose, which involves a secondary active, typically Na^+-dependent, transporter at the brush border membrane and a facilitative transporter at the basolateral membrane. Another possibility is that RFT2 might be modulated by an unknown mechanism and operate in a Na^+-dependent manner in the small intestine.

In conclusion, we have newly identified rRFT2 and also hRFT2. The finding of RFT2 is a significant and important step forward toward elucidation of the mechanisms of intestinal riboflavin absorption and its regulation, although future studies should be necessary to further clarify the role of RFT2 in the intestinal riboflavin absorption and unveil, if any, some other roles.

CONFLICT OF INTEREST

None declared.

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