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Differential expression of human riboflavin transporters -1, -2, and -3 in polarized epithelia: A key role for hRFT-2 in intestinal riboflavin uptake

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ABSTRACT

Transport of riboflavin (RF) across both the brush border membrane (BBM) and basolateral membrane (BLM) of the polarized enterocyte occurs via specific carrier-mediated mechanisms. Although, three human riboflavin transporters (hRFTs), i.e., hRFT-1, hRFT-2 and hRFT-3 are expressed in the intestine, little is known about the cell surface domain(s) at which these specific hRFTs are expressed. Here, we used live cell confocal imaging of intestinal epithelial Caco-2 and renal MDCK cells to show that the hRFT-1 is mainly expressed at the BLM, hRFT-2 is exclusively expressed at the apical membrane, while hRFT-3 is mostly localized inside intracellular vesicular structures (with some expression at the BLM). Further the level of hRFT-2 mRNA expression in Caco-2 cells and in native human intestine is significantly higher than that of hRFT-1 and -3; hRFT-2 was also more efficient in transporting ^{3}H-RF than hRFT-1 and -3. These findings implied an important role for hRFT-2 in intestinal RF uptake, a conclusion that was further supported by findings of hRFT-2 gene-specific siRNA knockdown investigation. These results show that members of the hRFT family are differentially expressed in polarized epithelia, and that the apically expressed hRFT-2 plays a key role in intestinal RF accumulation.

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1. Introduction

Riboflavin (RF; vitamin B2) is an essential micronutrient for normal cellular growth and function. In its coenzyme forms [riboflavin-5-phosphate and flavin adenosine dinucleotide], the vitamin plays key metabolic roles in biological oxidation-reduction reactions involving lipid, carbohydrate and amino acid metabolism, as well as in cellular metabolism of other water-soluble vitamins [1]. RF deficiency and sub-optimal RF levels occur in conditions like chronic alcoholism, diabetes mellitus, and inflammatory bowel diseases as well as in the elderly [2–6]. Deficiency of RF leads to a variety of clinical abnormalities that include neurological disorders, anemia, growth retardation, and skin abnormalities [1,7] as well as an increased susceptibility to cancer [8].

Mammals, including humans, have lost the capability for *de novo* synthesis of RF and obtain the vitamin from exogenous sources via intestinal absorption. The human intestine encounters RF from two sources: a dietary source (processed and absorbed in the small intestine), and a bacterial source from the normal large intestine microflora (absorbed in the large intestine) [9–13]. The mechanism of absorption of free RF has been studied using a variety of human and animal intestinal preparations [reviewed in Ref. 14]. Collectively, these studies have shown that absorption of RF in the small and large intestine is mediated by an efficient and specific, carrier-mediated mechanism. Since absorption of a nutrient across polarized intestinal epithelial cells represents movement across two functionally and structurally distinct membrane domains [brush border membrane (BBM) and basolateral membrane (BLM) domains], studies have also examined the mechanism(s) of RF transport across the individual membrane domain using purified membrane vesicles with results showing a specific carrier-mediated mechanism existing at each membrane domain [15–18]. Recently, molecular identity of the RFT systems that operate in human tissues has been defined. Three human riboflavin transporters, hRFT-1, hRFT-2 and hRFT-3 have been cloned [19–21] and all were shown to be expressed in the human intestine [19]. hRFT-1 and -3 share 87% identity at amino acid levels, whereas hRFT-2 shares 43 and 44% identity with hRFT-1 and -3, respectively [19]. Little, however, is known about the membrane domain(s) of the polarized enterocyte at which these different transporters are

Abbreviations: LAMP1, lysosome associated membrane protein1

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localized. Addressing this issue is crucial for understanding the details of the intestinal RF absorption process. Here, we investigated this issue using live cell confocal imaging in confluent monolayers of a polarized intestinal epithelial Caco-2 and renal epithelial MDCK cell types. Our findings demonstrate that hRFT-1 is mainly expressed at the BLM, while expression of the hRFT-3 occurs predominantly in intracellular vesicles (with some being at the BLM). Expression of the hRFT-2, on the other hand, occurs exclusively at the apical membrane domain [21,22]. These findings together with functional data involving both over-expression and selective knockdown with gene-specific siRNA, suggest a predominant role for the hRFT-2 in intestinal RF absorption.

2. Materials and methods

2.1. Materials

GFP-C3 and DsRed-C1 fluorescent protein vectors were from Clontech (Palo Alto, CA). LAMP1-RFP was obtained from Addgene Inc (Cambridge, MA). Caco-2, HuTu-80 and MDCK cells were from ATCC (Manassas, VA). ^{3}H-RF (specific activity ~12.3 Ci/mmol) was from Moravek Biochemicals (Brea, CA). DNA oligonucleotides primers (Table 1) were synthesized by Sigma Genosys (Woodlands, TX). hRFT-2 gene-specific siRNA was from Invitrogen (Carlsbad, CA; Table 1).

2.2. Generation of fluorescent protein fusion constructs

The full-length GFP-hRFT1, GFP-hRFT2, DsRed-hRFT2 and GFP-hRFT3 were generated by PCR amplification using hRFT-1, -2, and -3 gene-specific primer combinations (Table 1), PCR amplification kit (Clontech, CA) and PCR conditions as described previously [22–24]. The amplified PCR products for hRFT-1 and -3 and GFP-C3 vector were digested with *Hind III* and *Sac II*. The PCR product for hRFT-2, DsRed-C1 and GFP-C3 vectors were digested with *XhoI*/*SalI* and *BamHI*. The digested products were ligated separately for each construct using rapid DNA ligation kit (Roche Diagnostics, Indianapolis, IN) to generate in-frame fusion construct with GFP-C3 or DsRed-C1 fused to NH_2- terminus of each full-length construct. The generated fusion constructs were all verified by sequencing (Laragen, Los Angeles, CA).

2.3. Cell culture and transient transfection

Caco-2, HuTu-80 and MDCK cells were maintained in minimal essential medium [(MEM) ATCC, Manassas, VA]. MEM was supplemented with fetal bovine serum, glutamine, $NaHCO_3$ and antibiotics as described before [22–24]. Cells were grown on sterile glass-bottomed poly-D-lysine coated Petri dishes (MatTek, MA) or on 12 well plates (Corning, NY) and transfected with GFP, GFP-hRFT1, GFP-hRFT2, DsRed-hRFT2 and GFP-hRFT3 at 95% of confluency with 2-4 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen, CA) [22,25]. After 24–48 h of transient transfection, cells were imaged on Petri dishes or used for ^{3}H-RF uptake as described below.

2.4. Confocal imaging of fluorescent protein fusion constructs in live cells

Confluent cell monolayer was imaged using a Nikon C-1 confocal microscope. Green fluorescent protein (GFP) was excited with 488 nm line from an argon ion laser and emitted fluorescence was monitored with a 515 ± 30 nm short pass filter (GFP), Red fluorescent protein (DsRed) was excited with the 543-nm line from an HeNe ion laser and emitted fluorescence was monitored with a 570 ± 50 nm long pass filter. Images were captured using Nikon C-1 software (Nikon Instruments Inc, NY) [22,25].

2.5. siRNA treatment

hRFT-2 gene-specific siRNA was obtained from Invitrogen (Carlsbad, CA) with a sequence as listed in Table 1. Caco-2 cells pretreated with (60 nM) hRFT-2 siRNA or control siRNA (scrambled siRNA) for 48 h and were used for ^{3}H-RF uptake and for RNA isolation to perform real-time PCR analysis as described below.

2.6. ^{3}H-RF uptake assay

^{3}H-RF uptake assays were performed on GFP-hRFT1, GFP-hRFT2 and GFP-hRFT3 transiently expressing HuTu-80 cells, or hRFT-2 gene-specific siRNA pretreated Caco-2 cells in Krebs-Ringer buffer (pH 7.4) containing ^{3}H-RF (0.025 μM) as described previously [22,25]. Protein content of the cell digest was determined in parallel wells using protein assay kit (Bio-Rad, CA).

2.7. Real-time PCR analysis

Native human small intestinal RNA [pooled from 5 individuals (Clontech, CA)] or RNA isolated from Caco-2 cells was used for real-time PCR analysis. cDNA was synthesized from RNA using a reverse transcriptase kit (Invitrogen, CA) and real-time PCR was performed utilizing hRFT-1, -2, -3 and β-actin selective primers as described before [22,26,27].

Table 1
Combination of PCR primers used to prepare the full-length hRFT-1,-2 and -3 constructs of by PCR.

Construct	Forward & Reverse Primers (5'-3')	Positions Fragment	
		(bp)	(bp)
GFP-hRFT1[1-448]	CCC***AAGCTT***ATGGCAGCACCCACGCT; TCC**CCGCGG**GGGGCCACAGGGGTCTAC	1-1344	1344
GFP-hRFT2[1-469]	CCG*CTCGAG*ATGGCCTTCCTGATGCAC; CGGGATCCGGCTGGACAGTGCAGATTGCA	1-1407	1407
DsRed-hRFT2[1-469]	GC**GTCGAC**ATGGCCTTCCTGATGCAC; CGGGATCCGGCTGGACAGTGCAGATTGCA	1-1407	1407
GFP-hRFT3[1-455]	CCC***AAGCTT***ATGGCAGCACCCACGCCCGCC; TCCCCGCGGGGAGTCACAGGGGTCTGCACA	1-1365	1365
Real-time PCR primers			
hRFT-1	AAAAGACCTTCCAGAGGGTTG; AGCACCTGTACCACCTGGAT		
hRFT-2	CCTTTCCGAAGTGCCCATC; AGAAGGTGGTGAGGTAGTAGG		
hRFT-3	CCCTGGTCCAGACCCTA; ACACCCATGGCCAGGA		
β-actin	AGCCAGACCGTCTCCTTGTA; TAGAGAGGGCCCACCACAC		
hRFT-2 si RNA sequence			
CCG GCG CAC CUG UUC AUC UAU A; UCC UGC CUA ACA GGU CUC UGC UGU U			

Table 1 shows the primer sequences and combinations used to generate each construct. Restriction sites *Hin*dIII (bold face italics text) and *Sac*II (boldface underlined text for hRFT-1 and -3 and *Xho*I (boldface text)/*Sal*I (italics), and *Bam*HI (underlined text) for hRFT-2 were added to the primers to allow subsequent sub-cloning into the GFP-C3 or DsRed-C1vectors.