Differentiation-dependent regulation of intestinal vitamin B₂ uptake: Studies utilizing human-derived intestinal epithelial Caco2 cells and native rat intestine

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Running title: Regulatory aspects of intestinal riboflavin uptake

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Abstract

Intestinal epithelial cells undergo differentiation as they move from the crypt to the villi, a process that is associated with up- and down-regulation in expression of a variety of genes including those involved in nutrient absorption. Whether the intestinal uptake process of vitamin B₂ (riboflavin, RF) also undergoes differentiation-dependent regulation and through which mechanism is not known. We addressed these issues using the human-derived intestinal epithelial Caco-2 cells and native rat intestine as models. Caco-2 cells showed a significantly higher carrier-mediated RF uptake in post-confluent compared to pre-confluent cells. This up-regulation was associated with a significantly higher level of protein and mRNA expression of the RF transporters hRFVT-1 and hRFVT-3 in the post-confluent compared to pre-confluent cells; it was also accompanied with a significantly higher rate of transcription of the respective genes (i.e., SLC52A1 and SLC52A3, respectively) as indicated by the higher level of expression of the hnRNA and by a higher promoter activity in the former compared to the later cell types. Studies with native rat intestine also showed a significantly higher RF uptake by epithelial cells of the villus tip compared to those of the crypt; this again was accompanied with a significantly higher level of expression of the rat RFVT-1 and RFVT-3 at the protein, mRNA and hnRNA levels. These findings show for the first time that the intestinal RF uptake process undergoes differentiation-dependent up-regulation, and suggest that this is mediated (at least in part) via transcriptional mechanisms.

Key words: Intestinal transport, cell differentiation, riboflavin, RFVT-1, RFVT-3
Introduction

Vitamin B$_2$ (riboflavin; RF) is an essential micronutrient needed for normal cellular functions growth and development. In its biologically active forms [riboflavin-5-phosphate (FMN) and flavin adenosine dinucleotide (FAD)], RF plays an important metabolic role in cellular oxidation-reduction reactions involving carbohydrate, protein and lipid metabolism (8). RF deficiency leads to serious clinical abnormalities that include degenerative changes in the central nervous system, anemia, growth retardation, and skin lesions (8, 14, 28, 33). Deficiency and sub-optimal levels of RF have been observed in a variety of conditions as in patients with inflammatory bowel diseases, chronic alcoholism, diabetes mellitus, and chronic users of certain psychotropic agents (5, 12, 19, 30-32). RF deficiency also occurs in patients with Brown-Vialetto-Van Laere syndrome, a neurological disorder that is caused by mutations in hRFVT-3, as well as in patients with esophageal squamous cell carcinoma and gastric cardia adenocarcinoma (1, 6, 9, 24, 38, 45). Supplementation of RF to these patients brings a significant improvement in symptoms and biochemical abnormalities (1, 6, 16, 45).

Humans and other mammals cannot synthesis RF endogenously, and therefore, must obtain the micro-nutrient from exogenous sources via intestinal absorption. The intestinal epithelium, therefore, plays an essential role in determining and regulating the normal level of RF in the body. Previous studies from our laboratory and others have utilized a variety of human and animal intestinal preparations and showed that the intestinal absorption process of dietary RF in the small intestine is via a specific carrier-mediated mechanism [reviewed in (34, 35)]. The molecular identity of the systems involved in this absorptive event has only begun to emerge recently as a result of cloning of the RF transporters (RFVT-1, RFVT-2 and RFVT-3) from a variety of human and animal tissues (48-50). The hRFVT-1 and hRFVT-3 share 43 % identity at
the amino acid level with one another, while the hRFVT-2 shares around 87 and 44% identity with hRFVT-1 and -3, respectively (49). The RFVT-1 and RFVT-3 (the products of the SLC52A1 and SLC52A3 genes, respectively) are expressed at the basolateral and the brush border membrane domains of the polarized enterocytes, respectively, and appear to facilitate in the vectorial transport of RF from the lumen into circulation (40).

Intestinal epithelial cells undergo differentiation, which transforms the cells from an immature (undifferentiated) state to mature (differentiated) state. This event occurs as the cells move upward from their place of birth in the crypt to the villus region, and is associated with up- and down-regulation in expression of a variety of genes including those involved in nutrient (vitamin) absorption (7, 11, 22, 23, 25, 26, 39, 47). This differentiation-dependent regulation in expression of membrane carriers is designed to achieve and maintain normal function of intestinal epithelia. Understanding the mechanisms involved in regulation may assist in the designing of effective strategies to promote faster recovery of intestinal epithelia injury that occurs under certain patho-physiological conditions and as a result of use of certain pharmacological agents (e.g., certain anti-cancer drugs), therefore, addressing this issue in the case of RF is of physiological significance and may have therapeutic potential. There is nothing known about possible regulation of intestinal RF uptake during differentiation and the molecular mechanism involved, and thus, was investigated in this study. We use as models the intestinal epithelial Caco-2 cells [which differentiate spontaneously in culture upon reaching confluence; (4, 7, 17)]; and native rat intestine. Our results showed that the intestinal RF uptake process is up-regulated as the intestinal epithelial cells move from the undifferentiated to the differentiated state and that this differentiation-dependent regulation is mediated, at least in part, via transcriptional regulatory mechanism(s) involving the SLC52A1 and SLC52A3 genes.
Materials and Methods

Materials: $[^3]$H-RF (specific activity 21.2 Ci/mmol; radiochemical purity 98%) was obtained from Moravek Biochemicals (Brea, CA). Oligonucleotide primers were obtained from Sigma Genosys (The Woodlands, TX). All other chemicals and reagents used in this study were of analytical/molecular biology grade and were obtained from commercial sources.

Caco-2 cell culture and RF uptake assay: The human-derived intestinal epithelial Caco-2 cells, a well established model for studying differentiation-related aspects of intestinal epithelia (7, 11, 22, 23, 25, 26, 39, 47), were obtained from American Type Culture Collection (Manassas, VA) and were grown in a Modified Eagle’s medium (MEM, ATCC) supplemented with 10% (v/v) fetal bovine serum and appropriate antibiotics. Cells were plated at a density of 2 X $10^5$ cells/well onto 12-well plates (Corning Inc., NY). Uptake assays [initial rate, 3 min (36)] were performed on pre-confluent (1 day after seeding) and post-confluent (5 days after seeding, i.e., 3 days after confluence) Caco-2 cells. $[^3]$H-RF uptake was measured at 37°C in Krebs-Ringer buffer (pH 7.4) as described previously (26, 36, 39). Total protein content was determined using protein assay kit (Bio-Rad).

Isolation of villus and crypt cells from rat small intestine and uptake assay: Villus and crypt intestinal epithelial cells were isolated from the proximal half of rat small intestine as described before (26, 37, 46) using an established fractionation procedure (29). In this fractionation method, 10 factions were collected with fractions 1 and 2 representing upper villus (mature/differentiated) epithelial cells, while fractions 9 and 10 representing crypt (immature/undifferentiated) cells. Purity of these villus and crypt fractions has been established previously using marker enzymes approach (alkaline phosphatase and thymidine kinase for villus
and crypt epithelial cells, respectively) (26). Initial rate of \(^{3}H\)-RF uptake by freshly isolated villus and crypt cells was examined at 37°C in Krebs-Ringer buffer at pH 7.4 utilizing a rapid-filtration method (18). All animals used in this study received humane care in compliance with the American Association for Accreditation of Laboratory Animal Care, and the study was conducted according to protocols approved by the Veterans Affairs Medical Center- Long Beach Subcommittee of Animal Studies.

**Western blot analysis:** Total protein was isolated from pre-confluent and post-confluent Caco-2 cells as well as from rat small intestinal crypt and villus cells using RIPA buffer (Sigma). Proteins (60 μg) were resolved onto premade 4-12% Bis-Tris minigel (Invitrogen, CA), and subjected to western blot analysis as described previously (27, 43). After electrophoresis, proteins were electro-blotted onto Immobilon polyvinylidene difluoride membrane (PVDF) (Fisher Scientific, CA). Along with blocking buffer (LI-COR Bioscience, Lincoln, NB), the membranes were also incubated overnight either with human riboflavin transporters [hRFVT-1 (Abnova, CA), hRFVT-3 (Thermo Fisher Scientific, AL)] and rat riboflavin transporters [rRFVT-1 (Sigma, MO) and rRFVT-3 (Santa Cruz Biotechnology, CA)] specific polyclonal antibodies along with the respective β-actin monoclonal antibody (Santa Cruz, CA). Specificity of the rat and human RFVT-1 bands has been previously established by over expressing a GFP-RFVT-1 construct in cells, followed by using a GFP monoclonal antibody (data not shown). Specificity of the rRFVT-3 band was determined by using antigenic peptide (Santa Cruz, CA) (data not shown) and for hRFVT-3 as described before (27). The immunoreactive bands were detected and quantified as described before (27, 43).
**Real-time PCR:** Total RNA was isolated from pre-confluent and post-confluent Caco-2 cells, as well as from rat jejunum villus and crypt cells. The DNase I treated RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio Rad, CA) and real-time PCR was performed using combinations of gene specific primers (hRFVT-1, hRFVT-3, rRFVT-1, rRFVT-3, β-actin and 18S, Table1). Real-time PCR and determination of relative level of mRNA expression of hRFVT-1 and RFVT-3 in pre-confluent and post confluent Caco-2 cells, and that of the rRFVT-1 and RFVT-3 in rat intestinal villus and crypt cells were performed as described before (21).

**Generation of hRFVT-3 (SLC52A3) full-length promoter construct and assay of promoter activity:** The transcriptional start site for the hRFVT-3 gene was determined by mean of 5’-RACE (rapid amplification of cDNA ends) using a kit from Life Technologies (RACE version 2.0 kit, Invitrogen, CA). Genome analysis for hRFVT-3 reveals that it has one 5’-untranslated exon, and the putative ATG translational start site is located in the 2nd exon. In order to determine the transcriptional start site, the total RNA was isolated from Caco-2 cells and 1st strand cDNA was synthesized using gene specific antisense primer (5’- TAGAGACACGCTAAC-3’) which is complementary to nucleotides 88-73 bp upstream from the putative ATG start of translation codon in mRNA. The 1st strand was isolated and dC tailed following manufacturer’s protocol. The PCR was performed using abridged anchor primer (provided with the kit) and a second gene-specific reverse primer (5’-ATGGGAGACGAGGCTCGGAAG-3’) which is complementary to nucleotides 109-132 upstream from the ATG codon. The PCR product was further subjected to nested PCR using third gene-specific primer (5’-GCTCTCCTGGGATCTGGACTG-3’) complementary to nucleotides 142 -165 upstream of ATG codon. The PCR product was cloned in pGMT-easy vector and sequenced (Laragen, CA). We identified the hRFVT-3 transcriptional start site located 2710 bp upstream of ATG codon. We cloned the region 5832 to 2698 bp...
upstream of ATG codon in pGL3 basic vector using human genomic DNA (Clontech, CA) and gene specific primers (Table 1) designed to amplify a ~3.1 kb fragment of DNA including the transcriptional start site. Gene specific sequence was obtained from GenBank (gi 306518672 for hRFVT-3). The PCR amplification was performed as described before (39), gel purified using a gene clean II kit (MP Biomedicals, OH), and then sub-cloned into the TA vector (Promega, Madison, WI). This was subsequently sub-cloned into the promoter-less pGL3-basic vector (Promega, Madison, WI) using enzymes Mlu I and Hind III for hRFVT-3. Sequence of the hRFVT-3 promoter was verified by sequencing (Laragen, CA). The hRFVT-3 full-length promoter-luciferase reporter construct (4 µg) along with 100 ng of the transfection control plasmid Renilla luciferase-thymidine kinase (pRL-TK) (Promega, Madison, WI) were transiently co-transfected into Caco-2 cells grown in 12-well plates at less than 50% confluence using Lipofectamine 2000 reagent (Invitrogen). Caco-2 cells were then lysed 24 hrs after transfection (pre-confluence), and at 3 days after reaching confluence (post-confluence), and Renilla-normalized firefly luciferase activity was measured using the Dual Luciferase Assay kit (Promega, Madison, WI) and luminometer (Sunnyvale, CA)(26, 39).

Heterogeneous nuclear RNA (hnRNA) analysis: Level of hnRNA is used as a measure of transcriptional activity of a given gene under different conditions (2, 10, 20, 41, 42). The RNA samples were treated with DNase I to avoid amplification of genomic DNA and reverse transcribed with the random hexamer (Invitrogen). We determined the level of expression of human and rat RFVT-1 and RFVT-3 hnRNA in pre- and post-confluent Caco-2 cells and in rat villus and crypt epithelial cells using cDNA (synthesized from RNA) and gene-specific hnRNA primers that anneal to sequences in the intron/exon boundaries (Table 1). Real-time and semi-quantitative PCR were performed as described previously (41, 42). The real-time PCR data for
hRFVT-1 and hRFVT-3 were determined as described before (41, 42). The amplified rRFVT-1 and rRFVT-3 and β-actin hnRNA products were analyzed by agarose gel electrophoresis and the appropriate bands were quantified using UN-SCAN-IT gel digitizing software (Silk Scientific, Inc, Orem, UT).

**Statistical analysis:** All uptake data presented in this paper are mean ± SEM of multiple separate uptake determinations and are expressed in fmol/mg protein/unit time. Student’s t-test was used for statistical analysis; p < 0.05 was considered as statistically significant. Uptake of $^3$H-RF by the carrier-mediated system was determined by subtracting uptake by passive diffusion [represented by residual uptake of $^3$H-RF in the presence of a high pharmacological concentration of unlabeled RF (1 mM) from total $^3$H-RF uptake. Western blotting, real-time PCR, hnRNA, and promoter experiments were performed on at least three different occasions using different sample preparations.

**Results**

**A. Effect of differentiation on physiological and molecular parameters of RF uptake by human-derived intestinal epithelial Caco-2 cells:**

Figure 1 depicts the effect of differentiation of Caco-2 cells on the initial rate of carrier-mediated $[^3]$H-RF (14 nM) uptake. A significantly (p < 0.01) higher carrier-mediated RF uptake was observed in post-confluent (differentiated) Caco-2 cells compared to pre-confluent (undifferentiated) cells.

Figure 2 shows the effect of differentiation on the expression level of hRFVT-1 and hRFVT-3 proteins in Caco-2 cells. We focused on hRFVT-1 and hRFVT-3 since they represent the systems that transport RF across the basolateral and apical membrane domains of the
enterocyte, respectively (40). The results showed that the level of expression of the hRFVT-1 and hRFVT-3 proteins to be significantly (p < 0.05 and p < 0.01, respectively) higher in post-confluent compared to pre-confluent Caco-2 cells (Fig. 2A and B). In another study, we examined the effect of differentiation on the level of expression of the hRFVT-1 and hRFVT-3 mRNA and observed a significantly (p < 0.05 and p < 0.01, respectively) higher expression of both transporters in post-confluent compared to pre-confluent Caco-2 cells (Fig. 3A and B).

To explore the effect of differentiation on transcriptional activities of the \textit{SLC52A1} and \textit{SLC52A3} genes (which encode hRFVT-1 and hRFVT-3, respectively), we examined the level of hnRNA of hRFVT-1 and hRFVT-3 (by mean of real-time PCR) in pre-confluent and post-confluent Caco-2 cells. The hnRNA represents the first product of gene transcription and its level reflects transcriptional activity of the particular gene (2, 10, 20, 41, 42). The results showed a significantly (p < 0.01 for hRFVT-1, and p < 0.05 for hRFVT-3) higher level of expression of the hRFVT-1 and hRFVT-3 hnRNA in post-confluent compared to pre-confluent Caco-2 cells (Fig. 4A and B, respectively). These results suggest that the differentiation-dependent up-regulation in the expression of hRFVT-1 and hRFVT-3 is, at least in part, mediated by an increase in the transcriptional activity of the respective gene. To further confirm this, and focusing on the hRFVT-3 system since it is the predominant and most efficient RF transport system expressed in the human small intestine and appears to play a key role in intestinal RF uptake (40), we cloned and sequenced the 5'-regulatory region (~3.1 kb) of its gene (i.e., \textit{SLC52A3}). We then examined the effect of differentiation of Caco-2 cells on the activity of the \textit{SLC52A3} promoter (attached to the firefly luciferase reporter gene) after transient transfection into Caco-2 cells. The results showed a significantly (p < 0.01) higher \textit{SLC52A3} promoter activity in post-confluent compared to pre-confluent Caco-2 cells (Fig. 4C). This finding further
supports the suggestion that the increase in expression of RF transporters with differentiation is mediated, at least in part, via transcriptional mechanism(s).

**B. Effect of differentiation on physiological and molecular parameters of RF uptake by native rat intestine: Studies using freshly isolated intestinal crypt and villus epithelial cells:**

To validate the physiological relevance of the above findings with cultured Caco-2 cells, we used native rat intestine in these investigations. Immature crypt and mature villus epithelial cells were isolated from rat proximal small intestine and were used fresh to study initial rate of carrier-mediated $^3$H-RF (24 nM) uptake (36). The results showed a significantly (p < 0.05) higher carrier-mediated RF uptake in intestinal villus compared to crypt epithelial cells (Fig. 5).

We also examined the level of expression of the rRFVT-1 and rRFVT-3 proteins in rat intestinal crypt and villus cellular preparation by means of western blotting using specific polyclonal antibodies. The results showed a significantly (p < 0.05 and < 0.01, respectively) higher level of expression of the rRFVT-1 and rRFVT-3 proteins in intestinal villus compared to crypt epithelial cells (Figure 6A and B).

Level of expression of rRFVT-1 and rRFVT-3 mRNA in rat intestinal crypt and villus epithelial cells was also determined by mean of PCR with the results showing a significantly (p < 0.05 for both) higher level of expression of rRFVT-1 and rRFVT-3 mRNA in intestinal villus compared to crypt epithelial cells (Fig. 7A and B). Finally, we examined the level of expression of rRFVT-1 and rRFVT-3 hnRNA in rat intestinal crypt and villus epithelial cells to gain an insight into the transcriptional activities of the *Slc52a1* and *Slc52a3* genes. This was done by mean of semi-quantitative PCR with the results showing a significantly (p < 0.01 for both) higher level of expression of rRFVT-1 and rRFVT-3 hnRNA in villus compared to crypt
epithelial cells (Fig. 8A and B, respectively). These findings with native rat intestine again suggest that the differentiation-dependent regulation in intestinal RF uptake is mediated, at least in part, via transcriptional mechanism(s).

**Discussion**

Our aim in this investigation was to study whether the intestinal RF uptake process undergoes differentiation-dependent regulation, and if so, to shed light onto the physiological and molecular mechanisms involved in such a regulation. It is well established now that differentiation of intestinal epithelial cells is associated with changes in levels of expression of many genes including those that are involved in nutrient (vitamins) uptake (11, 23, 26, 39). We used the human-derived cultured intestinal epithelial Caco-2 cells in our investigations since they differentiate spontaneously in culture after reaching confluence and acquire a phenotype that closely reflects the mature absorptive enterocyte (4, 7, 17); indeed a large number of studies have used these cells in such type of investigations (7, 23, 26, 39, 44, 47). We complemented our findings using this cultured cell model with studies utilizing freshly isolated rat native intestinal epithelial cells.

Our studies with Caco-2 cells showed a significantly higher RF uptake by post-confluent (differentiated) compared to pre-confluent (undifferentiated) cells, clearly suggesting that the uptake process is under differentiation-dependent type of regulation. This was associated with a higher level of expression of the hRFVT-1 and hRFVT-3 at the protein and mRNA levels in the former compared to the latter cell-type. The latter findings suggest possible involvement of a transcriptional mechanism(s) in mediating, at least part of, the differentiation-dependent effect on intestinal RF uptake process. The findings that the level of expression of the hnRNA of
hRFVT-1 and hRFVT-3 is higher in post-confluent compared to pre-confluent Caco-2 cells supports this suggestion. This is because level of hnRNA (the first product of gene transcription) reflects transcription rate of the relevant gene, and thus, has been used as indicator for involvement of transcriptional mechanism(s) in changes in gene expression under different conditions (2, 10, 20, 41, 42). Further confirmation for the involvement of transcriptional mechanism(s) in mediating, at least part of, the differentiation-dependent regulation of intestinal RF uptake came from the finding on activity of the SLC52A3 full-length promoter expressed in pre- and post-confluent Caco-2 cells. In the latter study, a significantly higher SLC52A3 promoter activity was found in post-confluent compared to pre-confluent Caco-2 cells. Further studies are required to determine the differentiation-dependent responsive region in the SLC52A3 promoter of the RF transporter, and the cis- and trans-acting elements that mediate the differentiation-dependent effect.

The results with native rat intestinal epithelial cells complemented the findings with cultured Caco-2 cells in that a significantly higher carrier-mediated RF uptake was observed in intestinal villus (differentiated) compared to crypt (undifferentiated) cells. This was again associated with a higher level of expression of rRFVT-1 and rRFVT-3 at the protein and mRNA levels in the mature villus compared to the immature crypt epithelial cells. Also, the level of expression of rRFVT-1 and rRFVT-3 hnRNA was higher in villus compared to crypt epithelial cells. These findings again suggest possible involvement of a transcriptional mechanism(s) in mediating, the differentiation-dependent regulatory effect on intestinal RF uptake process.

Collectively, these findings demonstrate for the first time that the intestinal RF uptake process undergoes differentiation-dependent regulation, and suggests that this regulation is, at least in part, mediated via a transcriptional mechanism(s).
References


Acknowledgements

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Table 1. Combination of primers used to amplify the respective genes by real-time, semi-quantitative PCR and cloning of the promoters.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward and Reverse Primers (5’-3’)</th>
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<tr>
<td><strong>PCR Primers</strong></td>
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<tr>
<td>hRFVT-1</td>
<td>AAAAGACCTTCCAGAGGTTG; AGCACCTGTACCACCTGGAT</td>
</tr>
<tr>
<td>hRFVT-3</td>
<td>CCTTTCCGAAGTGCCCAC; AGAAGGTGAGGTAGTAGG</td>
</tr>
<tr>
<td>hβ-actin</td>
<td>AGCCAGACCCTCCTGGTA; TAGAGGGGCCCACACAC</td>
</tr>
<tr>
<td>rRFVT-1</td>
<td>AGCTACCTGTAGTGGTA; CTCAGCCCCTGAACCA</td>
</tr>
<tr>
<td>rRFVT-3</td>
<td>TAAGGAAGATCACGGCCACCT; GTCATCCAACGTGGCAACACAG</td>
</tr>
<tr>
<td>18S</td>
<td>GGGAGGTAGTGACGAAAAATAAACAAT; TTGCCCTCAATGGATCCTC</td>
</tr>
<tr>
<td><strong>hnRNA primers</strong></td>
<td></td>
</tr>
<tr>
<td>hRFVT-1</td>
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</tr>
<tr>
<td>hRFVT-3</td>
<td>TGACATCGCACAGG; CCTGCTGTGATCTG</td>
</tr>
<tr>
<td>hβ-actin</td>
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<td>rβ-actin</td>
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<td><strong>Primers used for cloning of hRFVT-3 promoter</strong></td>
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<td>hRFVT-3</td>
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Figure Legends

Figure 1. Uptake of \(^{3}\text{H}\)-RF by pre-confluent and post-confluent Caco-2 cells. Carrier-mediated (initial rate) uptake of \(^{3}\text{H}\) RF (14 nM) by pre-confluent and post-confluent Caco-2 cells was studied at 37°C in Krebs-Ringer buffer pH 7. Data are mean ± SEM from at least three independent uptake experiments from different batches of cells. * p < 0.01.

Figure 2. Level of expression of hRFVT-1 and hRFVT-3 proteins in pre-confluent and post-confluent Caco-2 cells. Total proteins (60 μg) isolated from pre-confluent and post-confluent Caco-2 cells were separated 4-12% mini gel and electro blotted to PVDF membrane and probed with specific anti-hRFVT-1 (A) and hRFVT-3 (B) polyclonal antibodies as described before (28, 45). Using LI-COR detection system as described in the “Methods”, the immunoreactive bands were detected and determined the specific band intensity. Data are mean ± SEM from at least three independent sample preparations from different batches of cells. * p < 0.01, ** p < 0.05.

Figure 3. Level of expression of hRFVT-1 and hRFVT-3 mRNA in pre-confluent and post-confluent Caco-2 cells. Real-time PCR was performed using hRFVT-1 (A) and hRFVT-3 (B) gene-specific primers and total RNA (5 μg) from pre-confluent and post-confluent Caco-2 cells as described in “Methods”. Data are mean ± SEM from at least three independent sets of experiments from different batches of cells and were performed on different occasions. * p < 0.01, ** p < 0.05.

Figure 4. Level of expression of hRFVT-1 and hRFVT-3 hnRNA in pre-confluent and post-confluent Caco-2 cells. Real-time-PCR was performed using hRFVT-1 (A) and hRFVT-3 (B) hnRNA gene-specific primers (Table 1) and RNA from pre-confluent and post-confluent Caco-2 cells as described in “Methods”. Data are mean ± SE of at least three independent determinations.
from three separate samples. C) Activity of full-length SLC52A3 (hRFVT-3) promoter in pGL3-
Basic was determined following transient transfection into Caco-2 cells. Luciferase activity was
determined in pre- and post-confluent Caco-2 cells and normalized relative to the activity of
simultaneously expressed Renilla luciferase as described in “Methods”. Data represents mean ±
SEM from at least three independent sets of determinations. * p < 0.01, ** p < 0.05.

Figure 5. Uptake of [3H] RF by freshly isolated native rat intestinal crypt and villus
epithelial cells. Carrier-mediated (initial rate) uptake of [3H]-RF (24 nM) by crypt and villus
intestinal epithelial cells isolated from rat jejunum was examined in Krebs-Ringer buffer pH 7.4.
Data represents mean ± SEM of at least three independent uptake determinations performed on
cells isolated from three different rats. ** p < 0.05.

Figure 6. Level of expression of rRFVT-1 and rRFVT-3 proteins in rat jejunal crypt and
villus cells. Total proteins (60 μg) isolated from crypt and villus cells were separated 4-12% mini
gel and electro blotted to PVDF membrane and probed with specific anti-rRFVT-1 (A) and
rRFVT-3 (B) polyclonal antibodies. The intensity of the immune-reactive bands was determined
as described above. Data are mean ± SEM of three separate determinations from three different
rats. * p < 0.01, ** p < 0.05.

Figure 7. Level of expression of rRFVT-1 and rRFVT-3 mRNA in native rat jejunum crypt
and villus epithelial cell. Real-time PCR was performed using rRFVT-1 (A) and rRFVT-3 (B)
gene specific primers (Table 1) and total RNA isolated from rat intestinal crypt and villus
epithelial cells. Data are mean ± SEM from at least three separate determinations on cells
isolated from three different rats. ** p < 0.05.

Figure 8. Level of expression of rRFVT-1 and rRFVT-3 hnRNA in rat jejunal crypt and
villus epithelial cell. Semi-quantitative RT-PCR was performed using rat RFVT-1 (A) and
RFVT-3 (B) hnRNA gene-specific primers (Table 1) and RNA isolated from rat intestinal crypt and villus cells as described in “Methods”. Data are mean ± SE of three separate samples from three rats. * p < 0.01.
Figure 1
Figure 2
Figure 3

Relative hRFVT-1 mRNA expression

A

Relative hRFVT-3 mRNA expression

B

Figure 3

Pre-confluent

Post-confluent

Pre-confluent

Post-confluent

**

*

0  5  10

0  6  12
A. hRFVT-1 hnRNA

B. hRFVT-3 hnRNA

C. hRFVT-3 promoter activity

Figure 4
Figure 5

Carrier-mediated RF uptake (fmol/mg protein/5 min)

Crypt          Villus

225

0

450

**
Figure 6
Figure 7
Figure 8