Involvement of a Receptor-Mediated Component in Cellular Translocation of Riboflavin

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ABSTRACT
This study addresses the transport mechanism of riboflavin (vitamin B₂) across intestinal epithelium in the presence and absence of pharmacologically active compounds. A polarized transport process with a 6-fold higher basolateral (BL)-to-apical (AP) flux was observed in both a human intestinal cell model (Caco-2) and rat intestinal tissue. Riboflavin-specific translocation systems on both the AP and BL cell surfaces were saturated with affinity values close to most receptors (Kᵢₒ: 9.72 ± 0.85 and 4.06 ± 0.03 nM, respectively). Pharmacological agents known to alter intracellular endocytic events were used to examine the potential involvement of receptor-mediated events. Nocodazole significantly inhibited AP uptake (58.4%), BL-to-AP riboflavin (56.7%) and fluorescein isothiocyanate-labeled transferrin (FITC-Tf) (31.8%) transport without affecting mannitol or cholic acid transport, whereas AP-to-BL riboflavin (252.8%) and FITC-Tf (145.1%) transport was increased. Brefeldin A significantly enhanced AP-to-BL riboflavin (37.1%) and bidirectional FITC-Tf transport (AP-to-BL: 13-fold; BL-to-AP: 5-fold), without affecting BL-to-AP riboflavin transport. Combined, these data suggest an essential role of microtubule-dependent movement and vesicular sorting component(s) in the bidirectional transport of riboflavin. Dissociation of riboflavin from the cell surface was pH-dependent with significantly higher substrate release at acidic pH, indicating the presence of riboflavin-specific cell surface receptors. In summary, our studies provide biochemical evidence of the involvement of a receptor-mediated mechanism in the cellular translocation of riboflavin.

The cell membrane imposes a formidable absorption barrier to the translocation of water-soluble vitamins. To accommodate the entry of these essential nutrients, the cell expresses specific membrane proteins on the cell surface. These proteins are part of a specialized uptake mechanism that can be broadly categorized as carrier-mediated and receptor-mediated endocytosis (RME). The first mechanism facilitates the movement of vitamin molecules across the cell membrane via membrane carrier protein(s) energized by ATP hydrolysis or the cotransport of ions moving down their electrochemical gradient. In fact, transport of most B vitamins has been identified to occur via a Na⁺ or H⁺-dependent carrier-mediated pathway (Dutta et al., 1999). In the second model, the vitamin molecules may first bind to an endogenous protein that in turn binds to surface receptors (e.g., vitamin B₁₂) or directly binds receptors localized in specialized membrane regions (e.g., folate) before they are internalized into endocytic vesicles (Antony, 1996).

Cellular uptake of riboflavin, also known as vitamin B₂, has been extensively investigated in a variety of cell lines, and organs and tissues (intestine, liver, and kidney) from several species (human, rat, and rabbit) (Said and Arianas, 1991; Rindi and Gastaldi, 1997). From these studies, it appears that riboflavin is taken up into most cells via an active, carrier-mediated mechanism that is pH-independent. It has been suggested that these mechanisms are present on both surfaces of epithelial cells (Said et al., 1993; Said and Mohammadkhani, 1993). Contradictory results have been reported regarding the influence of Na⁺ on riboflavin uptake. Although some studies suggest no obvious requirement for Na⁺ or partial Na⁺ dependence (Middleton, 1990; Said and Arianas, 1991), most reports indicate riboflavin transport to be Na⁺-independent (Said and Ma, 1994; Dyer and Said, 1995). Furthermore, riboflavin uptake is not sensitive to the Na⁺/K⁺-ATPase inhibitor ouabain (Said and Ma, 1994; Dyer and Said, 1995), confirming the Na⁺-independent behavior of this transport system. The apparent Na⁺, K⁺, and H⁺ (pH) independence of the riboflavin transport system would classify this system as a uniporter, challenging the current paradigm that mammalian apical (AP) solute carrier proteins are predominantly cotransporters (Hediger et al., 1995). The outlined controversy surrounding riboflavin absorption prompted us to explore alternative hypotheses to better rationalize the uptake mechanism of this important vitamin.

ABBREVIATIONS: RME, receptor-mediated endocytosis; FITC-Tf, fluorescein isothiocyanate-labeled transferrin; TIR, transferrin receptor; AP, apical; BL, basolateral; BFA, brefeldin A; Iₛₑ, short-circuit current.

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Another possibility for the active cellular uptake of riboflavin is internalization by surface receptors via a process similar to the RME of other water-soluble vitamins such as folate and vitamin B₁₂. In fact, a soluble high-affinity riboflavin-binding protein has been detected in plasma (Zheng et al., 1988) and the reproductive organs of either sex (Natraj et al., 1994), although its function in riboflavin transport remains to be defined. Interestingly, Low and coworkers recently showed that the facilitated entry of BSA into lung epithelial cells and other cell cultures after covalently coupling BSA to riboflavin (Wangensteen et al., 1996; Holladay et al., 1999). Conjugates were detected in endosomal compartments, suggesting that BSA-riboflavin conjugates enter the cell via endocytosis. However, direct evidence of the involvement of endocytosis and/or transcytosis in the uptake of riboflavin in any cell type has not been reported previously.

To elucidate the cellular translocation mechanism of riboflavin in the intestine and investigate the potential involvement of an RME component, we have determined its binding and transport in the absence and presence of pharmacologically active compounds that are known to affect vesicular trafficking pathways. We used the well characterized Caco-2 cell line as a model for the small intestine. These cells, when grown to confluence on polymer membrane inserts, mimic the in vivo intestinal absorption process, enabling us to characterize vectorial substrate movement across both cell membranes and the cytoplasm.

**Experimental Procedures**

**Materials**

[³H]Riboflavin (20 Ci/mmol) and [¹⁴C]mannitol (60 mCi/mmol) were purchased from Sigma (St. Louis, MO). [³H]Cholic acid (25 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Cell culture materials and buffer solutions were obtained from Life Technologies (Grand Island, NY). Transwell inserts were purchased from Costar (Corning, NY) and rat tail collagen (type I) was from Becton Dickinson Labware (Bedford, MA). Human transferrin (Tf), fluorescein isothiocyanate (FITC) conjugation kit, BCA protein assay kit, nocodazole, and brefeldin A (BFA) were purchased from Sigma. All other chemicals were from Fisher Scientific (Pittsburgh, PA).

**Cell Culture**

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Cells with passage numbers 23–38 were maintained at 37°C under 5% CO₂ in complete medium consisting of Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. Cells were plated at 6 × 10⁴ cells/cm² in tissue culture treated flasks and used during the exponential growth phase (day 5). Cells were propagated and cultured as described previously (Hidalgo and Borchardt, 1990). Cell monolayers were grown on collagen-coated polycarbonate Transwell membrane inserts (3.0-µm pore size) at a density of 63,000 cells/cm². The culture medium was changed every other day during the 1st week after seeding and daily thereafter. Protein content was determined by the BCA method using BSA as standard.

**Assessment of Transepithelial Membrane Resistance**

An epithelial voltohmometer with dual electrodes (World Precision Instruments, New Haven, CT) was used to measure transepithelial resistances of monolayers grown on filters as described by Rindler and Traber (1988). Filters were used only if the electrical resistance of the monolayer exceeded 250 Ω · cm². Coincubation with [¹⁴C]mannitol (0.2 µCi/ml) was performed as an independent confirmation that paracellular transport in monolayers with electrical resistances higher than 250 Ω · cm² was indeed minimal.

**Transepithelial Transport Studies (Caco-2 Cells)**

Before transport studies, cell monolayers were washed twice with warm Dulbecco’s PBS and incubated for 30 min at 37°C with substrate-free bathing medium (Hanks’ balanced salt solution containing 25 mM glucose and 10 mM HEPES, adjusted to pH 7.4). Transepithelial studies were initiated by adding 1.5 to 480 µl of 10 µCi/ml [³H]riboflavin and 30 µl of 10 µCi/ml [¹⁴C]mannitol stock solutions to the donor compartment of pre-equilibrated Caco-2 cell monolayers, thereby achieving final donor concentrations of 0.5 to 160 nM [³H]riboflavin and 3.6 µM [¹⁴C]mannitol. The donor compartment volume was adjusted accordingly to maintain a constant 1.5-ml AP bathing volume. The transepithelial flux of [³H]riboflavin and [¹⁴C]mannitol from either direction was followed over time by withdrawing samples at t = 0, 15, 30, 45, 60, 90, 120, 150, and 180 min. To maintain a constant volume, an identical volume of bathing medium was added back after every sample. Cell monolayers (14 days postseeding) that exerted a mannitol flux larger than 0.18%/h/cm² were excluded from data analysis (Hidalgo, 1996). At the end of the experiments, the AP and basolateral (BL) bathing media were removed. Cell monolayers were washed three times with ice-cold PBS, pH 7.4, and cells were scraped off the inserts and processed for liquid scintillation counting.

**Transport Studies with Rat Intestinal Tissue**

Three-month-old male Sprague-Dawley rats fed ad libitum were used in all experiments. The small intestine was removed after decapitation, and tissue was prepared for mounting in side-by-side diffusion chambers (Ussing chamber type) as described previously (Swaan et al., 1994). Briefly, mucosa was stripped of underlying muscle, mounted in the Ussing chamber (1-cm² exposed surface area), and bathed on both sides with Ringer’s buffer solution. Solutions were circulated by gas lift with carbogen (95%O₂, 5%CO₂; Liquid Carbonic, Columbus, OH) and maintained at 38°C (rat body temperature) by water-jacketed reservoirs. Tissues were equilibrated for 30 min before initiation of experiments. Potential difference (P_d) and short-circuit current (I_sc) were monitored during the entire transport experiments to ascertain tissue viability. Tissue integrity was independently determined by assessing the [¹⁴C]mannitol flux (Marks et al., 1991). Tissue viability was further assessed at the end of the study by spiking AP sides of tissue with a 1 M glucose solution (100 µl); the intestinal tissues with a 2- to 4-fold I_sc jump were considered viable. I_sc values ranged from 10 to −25 µEq/h · cm² to 40 to −125 µEq/h · cm² after addition of d-glucose.

**Binding Studies**

Caco-2 cells were seeded on 6-well plates and cultured for at least 14 days. Before experiments, cells were washed twice with ice-cold PBS. After incubation at 4°C for 1 h with 2 nM [³H]riboflavin, cells were washed twice with ice-cold PBS (6 ml/well for each 2-min wash) and surface-bound riboflavin was released by incubation with 1 ml of ice-cold PBS with pH values ranging from 3.0 to 8.0 for 2 min. After buffer collection, cells were washed again with 6 ml of ice-cold PBS and finally lysed with 0.5 ml of a 1% Triton X-100 solution. To ascertain mass balance, cell lysates and samples from each PBS washing step were analyzed for radiolabeled material. One nanomolar [¹⁴C]mannitol was incorporated in the incubation medium as a control for the specificity of the washing steps. Nonspecific binding and potential passive diffusion was determined in parallel studies by measuring radioactivity bound in the presence of a 1000-fold excess of nonradiolabeled riboflavin. Riboflavin-specific binding was obtained by subtracting nonspecific binding count from the total radioactivity.
Analytical Methods

Radioactivity. The amount of dual-labeled radioactivity in the samples was quantitated using a Beckman liquid scintillation counter (model LS 6000IC; Fullerton, CA) at a counting efficiency of 43 and 75% for \(^{3} \text{H}\) and \(^{14} \text{C}\), respectively.

HPLC analysis. A Beckman HPLC system, consisting of a model 166 UV detector and a LPSX gradient pump, was coupled in series with a Packard radiometric flow scintillation analyzer (Packard Instruments, Meriden, CT). A Merck reversed phase RP-C18 column (10 cm, 5 μm) was eluted at 3 ml/min with a scintillation cocktail and 1 ml/min with 87% 10 mM ammonium phosphate (pH 5.5)/acetonitrile (Pietta et al., 1982).

Data Analysis and Statistics

Uptake of riboflavin or cholic acid by cell monolayers was expressed as femtomoles per milligram of protein. Unidirectional flux was estimated over a period of 0 to 180 min with an observed lag time of approximately 10 to 15 min. Linearity was observed up to at least 180 min. Values in figures and tables are means ± S.D. of at least three different experiments with cells from different passages. The effect of sample withdrawal was taken into account for the calculation of fluxes using the following equation:

\[
Q = V_{n} \left( \sum_{a=1}^{n} C_{a} - 1 \right) + V_{1} C_{1}
\]

(1)

where \(Q\) is the total amount of radioactive ligand in the donor compartment, \(V_{n}\) is the sample volume, \(V_{1}\) is the volume of Transwell, and \(C_{1}, C_{2}, \ldots, C_{n}\) represents the concentration of sample 1, 2, \ldots, \(n\). Transport parameters [Michaelis-Menten constant \((K_{m}\)] maximum flux \((J_{\text{max}})\), and passive permeability coefficient \((P_{m})\) were calculated using the NONLIN module in SYSTAT (version 8.0, SPSS Inc., Chicago, IL) by nonlinear regression analysis of the obtained data to the general expression for transepithelial flux \((J)\):

\[
J = \frac{J_{\text{max}} \times C}{K_{m} + C} + P_{m} \times C
\]

(2)

Statistical analyses were performed by one-way ANOVA and significant differences were reported with a confidence interval of 95%.

Effect of Culture Time on Riboflavin Transport.

Within 10 days after achieving confluency, Caco-2 cells undergo a differentiation program that involves formation of tight junctions (as assessed by changes in electrical resistance across monolayers grown on filters) and marked elevations in the levels of several brush border hydrolases and transport proteins (Delie and Rubas, 1997). When grown on filters, this proliferation and differentiation program yields a functional monolayer of cells strongly resembling the small intestinal epithelium. It has been shown previously that the transepithelial transport level of some nutrients such as biotin and vitamin B\(_{12}\) across these monolayers can vary significantly with days in culture, depending on their specific membrane transport protein expression during differentiation (Dix et al., 1990; Ng and Borchardt, 1993). Thus, we first investigated the optimal culture time for maximal expression of riboflavin transport protein(s). In this experiment, we chose a \(^{3} \text{H}\)riboflavin concentration in accordance with the baseline riboflavin concentration in human plasma (~12 nM) (Zempleni et al., 1996). \(^{14} \text{C}\)Mannitol was used as a control to ascertain monolayer integrity. This compound uniquely diffuses across the cell membrane via the paracellular pathway, providing a quantitative measure of tight junctional development in maturing Caco-2 cell monolayers (Marks et al., 1991, Swaan et al., 1994).

A relatively high mannitol flux 7 days after seeding indicates that a cohesive monolayer has not yet been established (Fig. 1). This supports the premise that membrane leakiness accounts for the apparent maximal riboflavin flux in the 1st week postseeding. After 7 days in culture, however, no significant difference can be observed in the transepithelial flux of either riboflavin or mannitol in both transport directions with regard to culture time (AP-to-BL or BL-to-AP; \(P > .05\); one-way ANOVA). Accordingly, all subsequent transport studies were performed at or after 14 days in culture. The BL-to-AP riboflavin flux is consistently higher, approxi-

![Fig. 1. Effect of culture time on riboflavin transport in Caco-2 cell monolayers.](image-url)
Metabolic Stability of [3H]Riboflavin. In addition to formation of tight junctions, functional differentiation in Caco-2 cells is accompanied by elevated expression of several metabolic enzymes. Multiple studies have shown that enzymatic activities gradually increase during differentiation and reach maximal levels 15 to 21 days after confluency (reviewed by Delie and Rubas, 1997). It has also been suggested that phosphorylation of riboflavin inside enterocytes is an essential step of its AP absorption (Kasai et al., 1988). To investigate whether putative enzymatic conversion and metabolic instability of [3H]riboflavin (which contains a general tritium label) could contribute to the observed transport polarity, we analyzed samples from transport experiments on an HPLC system coupled to a radiometric liquid scintillation detector. Chromatograms of samples after a 3-h transport study (Fig. 2) indicated that most radiolabeled riboflavin on the donor side remains unchanged (Fig. 2, B and C), although we cannot exclude the possibility that any metabolites are below the limit of detection on this system. On the AP acceptor side, 85% of transported ligand remains in the form of unchanged riboflavin (Fig. 2A), whereas the remaining activity cannot be ascribed to the major coenzyme forms of riboflavin, FMN and FAD, which would elute at 2.5 and 3.0 min, respectively.

Polarization of Riboflavin Transport in Rat Tissue. Although the Caco-2 cell culture system is known to closely mimic small intestinal enterocytes, it has been reported that these cells exert slightly different biochemical indices and constitute altered protein composition and expression when compared with human small intestinal enterocytes. To further validate that the observed transport polarity of riboflavin did not reflect a cellular aberration inherent to the biochemical differences between Caco-2 cells and human intestinal epithelial cells in vivo, we conducted transport studies using rat intestinal tissues mounted in side-by-side diffusion chambers (Usinger chamber type). Figure 3 shows that riboflavin transport in rat intestine from both directions is linear up to 180 min with a significantly greater BL-to-AP flux (approximately 5-fold, 577.5 fmol/h/cm²; R² = 0.99) over AP-to-BL flux (124.9 fmol/h/cm²; R² = 0.98).

Concentration Dependence of Riboflavin Transport. The observed polarization in transepithelial transport of riboflavin could be attributed to the presence of two different transport systems located on the AP and BL sides of the membrane. To test this possibility, concentration-dependence experiments were carried out in both transport directions. Figure 4 demonstrates that riboflavin is transported by a binary mechanism consisting of both a saturable and a linear (passive) component. At concentrations near basal plasma riboflavin concentrations, riboflavin is transported predominantly by the saturable component. Table 1 lists the kinetic parameters of the transport systems on both sides of the epithelium. Consistent with our findings (previous sections) that riboflavin transport is greater in the BL-to-AP direction, we determined that a BL transport system had a 2-fold higher affinity (Km) compared with the AP translocation system. These results suggest that the higher BL-to-AP riboflavin transport is partly due to the higher affinity of riboflavin for the BL system.

Effect of Endocytosis Inhibitors on Riboflavin Transport. The affinity values (Km) for carrier-mediated transport pathways are generally in the micromolar range, whereas most receptor-mediated processes exert substrate affinity values in the low nanomolar range (Peenez and King, 1998). Concentration-dependence studies reveal two high-affinity riboflavin transport systems with Km values in the low nanomolar range (Table 1). Therefore, our results suggest the involvement of a receptor-mediated mechanism in the transepithelial transport of riboflavin. Moreover, most receptor-mediated transcytosis systems transport their substrates preferentially in the BL-to-AP direction (Okamoto, 1998). The interesting consistency between these observations and our current results led us to hypothesize that cellular uptake of riboflavin may use an RME/transcytosis mechanism similar to that of other vitamins in the B group, e.g., folate and vitamin B₁₂. Without the availability of a cDNA clone of the postulated transporter/receptor, it is relatively difficult to distinguish between RME and carrier-mediated pathways using contemporary biochemical or electrophysiological techniques because both mechanisms reveal characteristics of active transport processes. However, several inhibitors are known to affect specific processes and/or organelles involved in vesicle trafficking, protein sorting, RME, and transcytosis. To directly test our hypothesis, two inhibitors with different mechanisms of action were chosen: nocodazole and BFA. At the cellular level, nocodazole inhibits endocytosis by depolymerizing microtubules (Hamm-Alvarez and Sheetz, 1998), which are necessary for endocytic vesicles to move within the cell, and BFA induces missorting of vesicles in the trans-Golgi network (Wan et al., 1992).

To confirm the specificity of endocytosis inhibitors in distinguishing carrier-mediated processes from RME, we also examined the transepithelial transport of cholic acid (negative control) and Tf (positive control). Cholic acid is a known substrate of the intestinal bile acid transporter, a well-characterized carrier-mediated transport system (reviewed by Swaan, 1996). In Caco-2 cells, it has been demonstrated that cholic acid is absorbed via a bile acid transporter with a Km of 49.7 μM (Hidalgo and Borchardt, 1990). Furthermore, the transepithelial flux of cholic acid can serve as an indicator for functional integrity and viability of a Caco-2 cell culture system (Hidalgo, 1996). Tf, an iron transport protein internalized by the Tf receptor (TfR), is used as a positive control for endocytosis because the RME mechanism of TfR has been well established (reviewed by Huebers and Finch, 1987). Endocytosis of FITC-labeled Tf (FITC-Tf) has been characterized in different cell lines, and FITC-Tf is widely used as an endosome marker in microscopic analysis of an RME event (Teter et al., 1998). Thus, to visualize and detect the transport of Tf across the Caco-2 cell monolayer, a FITC-Tf conjugate was synthesized.

Figure 5 shows the effect of endocytosis inhibitors on bidirectional transepithelial transport of riboflavin, cholic acid, and FITC-Tf. Compared with the control, nocodazole inhibits BL-to-AP transport of riboflavin and FITC-Tf (56.7 and 31.8%, respectively) but does not affect cholic acid transport from both directions (Fig. 5A). AP-to-BL transport of riboflavin and FITC-Tf is significantly increased after treatment with nocodazole (Fig. 5B). Corresponding mannitol fluxes indicate that nocodazole does not compromise the intactness of Caco-2 cell monolayers during these transport studies.
Fig. 2. Stability of [3H]riboflavin in 14-day postseeding Caco-2 cells. AP and BL samples after a 3-h transport study were analyzed by HPLC with radiometric detection. [3H]Riboflavin elutes at 6.0 to 6.2 min, and [14C]mannitol has a retention time of 1.6 to 1.7 min. A, AP sample chromatogram from the 3-h BL-to-AP transport study. B, BL sample chromatogram from the 3-h BL-to-AP transport study. A yet-to-be-defined peak was detected in AP samples at 5.1 min. C, AP sample chromatogram from the 3-h AP-to-BL study.
Figure 5B demonstrates that after exposure of cell monolayers to BFA, AP-to-BL riboflavin flux increases 37.1%, but the transport of cholic acid is not affected. Polarized transport of FITC-Tf is observed in the control Caco-2 cells with a 6-fold greater BL-to-AP transport. BFA significantly enhances FITC-Tf transport in both directions (AP-to-BL: 13-fold; BL-to-AP: 5-fold). Elevated riboflavin fluxes are not the result of monolayer leakiness as evidenced by constant mannitol fluxes (control: 5.31 ± 1.33 pmol/h/cm²; BFA treated: 6.19 ± 1.33 pmol/h/cm²). Compared with control, the BL-to-AP transport of riboflavin is slightly decreased although not statistically different (P > .05), whereas cholic acid transport is not affected by BFA treatment (Fig. 5A). To investigate the effect of nocodazole on AP uptake of riboflavin without the interference of BL-to-AP backflux, we performed uptake studies using tissue culture plates. As shown in Fig. 5C, 58.4% of AP uptake of riboflavin is blocked by nocodazole within 20 min. The combined effects of nocodazole and BFA on both the transport and uptake of riboflavin demonstrated the involvement of an RME mechanism. This observation is further substantiated by the effects of these compounds on FITC-Tf and cholic acid.

**pH-Dependent Dissociation of Surface-Bound Riboflavin.** An additional distinct mechanistic feature of ligands for RME pathways is pH-dependent dissociation of these molecules from their receptors (reviewed by Mukherjee et al., 1997). Consequently, we performed surface binding experiments at 4°C using wash buffers with pH values of 3 to 8. At 4°C, the cellular internalization of riboflavin is significantly blocked (data not shown). Nonspecific binding is determined from parallel studies in the presence of a 1000-fold excess of unlabeled riboflavin, and [14C]mannitol binding serves as a negative control. A decrease in pH from 8 to 3 resulted in elevated dissociation of riboflavin from its specific binding site(s) with significantly greater amounts of riboflavin released at pH 3 and 4, whereas the release of mannitol is pH-independent (Fig. 6). At physiological pH of the small intestinal lumen (pH 6–7), riboflavin shows higher binding interaction to its cell surface receptors.

**Discussion**

Although multiple studies have characterized the *uptake* mechanism of riboflavin in a variety of tissues and species, there is surprisingly little information on the transepithelial *transport* of riboflavin across different cell types. In this study, we find that at concentrations around its *K*ₘ, riboflavin transport from the BL to the AP surface is 6-fold greater, compared with the flux in the AP-to-BL direction. This ele-
vated transport from the systemic circulation into gut lumen has not been observed previously either in cell culture systems (Fig. 1) or in intestinal tissue preparations (Fig. 3). The fact that transport polarity is conserved between two different species (rat versus human) further confirms the physiological and biological relevance of this observation. It also validates that the striking polarity of riboflavin transport observed in Caco-2 cells is not due to a transport anomaly specific to this particular cell line.

Interestingly, a similar transport polarity has been reported for intestinal Tf absorption (Shah and Shen, 1994) with a 40-fold difference in BL-to-AP versus AP-to-BL translocation activity and receptor expression. Enterocytes are known to obtain iron from dietary iron uptake and, during periods of low dietary iron intake, also from body iron stores. Analogous to intestinal iron homeostasis, we anticipate that intracellular riboflavin levels in the gut are tightly regulated as well. Therefore, the putative physiological function of the riboflavin receptor on the BL surface of enterocytes is to participate in the supply of riboflavin to maturing epithelial cells. Because the intestine has the highest cell turnover rate of any tissue, a continuous supply of riboflavin would be required to support cell growth and differentiation as well as the synthesis of essential flavoproteins such as digestive enzymes.

The $K_m$ values we reported from transport experiments (Table 1) are 100-fold lower than those reported by Said and coworkers, who found values in the low micromolar range ($0.3 \mu M$) via uptake studies (Said and Ma, 1994). It is essential, however, to point out two consequential differences between the previous studies and our data. First, transepithelial transport in polarized cells comprises a series of sequential events, namely ligand-binding, internalization, ligand movement through the cytoplasm, and translocation across two cell membranes with different lipid compositions. Uptake experiments can only discern binding and movement through the AP membrane. Second, we selected riboflavin concentrations according to the reported basal human serum
level (12 nM), whereas earlier studies cover higher concentrations in the micromolar domain. If more than one type of riboflavin transport system exists in Caco-2 cells, the higher affinity system would not be detected at ligand concentrations 10-fold greater than the $K_m$ value of this system. Therefore, the possibility exists that small intestinal cells adopt two different membrane transport mechanisms for riboflavin with unequal substrate affinity. Interestingly, this observation correlates with the previous discovery of two unique coexisting transport systems for folic acid, another member of the B-vitamin group. It is now widely accepted that the cellular uptake of this essential molecule is mediated by both a glycosylphosphatidylinositol-anchored receptor with a $K_m$ of 0.1 nM and a transmembrane carrier protein with an apparent $K_m$ of 1 $\mu$M (reviewed by Reddy and Low, 1998). These findings and our current observation may suggest that more unique high-affinity transport mechanisms could exist for other members of the B-vitamin group.

In intestinal epithelial cells, microtubules extend in straight arrays from the AP to the BL cell membrane, providing an essential network for many membrane-trafficking events including endocytosis and transcytosis (Hamm-Alvarez and Sheetz, 1998). Disruption of microtubules with nocodazole significantly inhibits BL-to-AP transport of riboflavin (Fig. 5A). These results are in good agreement with the data by Maples et al. (1997), who reported a 50 to 75% inhibition of the BL-to-AP transcytosis of polymeric IgA in Madin-Darby canine kidney cells in the presence of nocodazole. More importantly, nocodazole inhibition of the BL-to-AP flux of FITC-Tf, a well-characterized RME substrate, suggests that these two compounds share similar microtubule-dependent intracellular trafficking pathways. Specificity of nocodazole for RME processes, but not carrier-mediated events, was shown by the apparent lack of reduction in cholic acid flux. This observation, in turn, rules out the possibility that our data are a reflection of cellular damage caused by the adverse effects of nocodazole on protein synthesis and signal transduction (Hamm-Alvarez and Sheetz, 1998).

It has been reported that polarized epithelial cells have spatially separated early endosomal systems governing distinct bidirectional endocytosis/transcytosis processes (Mukherjee et al., 1997). These two pathways are also functionally distinct in their sensitivities toward pharmacological agents (Okamoto, 1998). Interestingly, nocodazole treatment results in increased AP-to-BL transport of both riboflavin and FITC-Tf (Fig. 5B). Currently, the factors underlying this phenomenon are not completely understood and need further investigation.

Even though AP and BL endosomal systems are separately regulated in epithelial cells, studies in Caco-2 cells have revealed an interconnection via intermediate endosomes and common recycling compartments (Knight et al., 1995). BFA is known to cause intracellular missorting of ligand-receptor complexes within the endosome-trans-Golgi network, a region closely involved in vesicular sorting and bidirectional endocytosis/transcytosis (Wan et al., 1992). In our studies, BFA results in a significant increase in AP-to-BL transport of riboflavin and FITC-Tf without perturbing the cholic acid flux. BL-to-AP riboflavin transport is slightly, but not significantly, reduced ($P > .05$). These results are consistent with previous studies showing that BFA induces RME-mediated transcytosis of $^{125}$I-Tf across Caco-2 cell monolayers in both transport directions (Shah and Shen, 1994). These observations are also in agreement with the findings that BFA treatment leads to enhanced transcytosis of ricin and Clostridium botulinum neurotoxin (Prydz et al., 1992; Maksymowych and Simpson, 1998). Shen and colleagues demonstrated that enhancement of Tf transcytosis by BFA is accompanied by a significant increase in the number of TfRs on the AP cell membrane and a decrease in the number of TfRs on the BL cell membrane without affecting total TfR expression (Wan et al., 1992; Shah and Shen, 1994). Whether a similar mechanism is responsible for increased riboflavin transport remains to be investigated.

Our finding that dissociation of riboflavin from its specific surface binding site(s) is pH-dependent (Fig. 6) provides additional biochemical evidence corroborating our hypothesis. This observation is consistent with a hallmark feature of RME in which acidification of endosomes during the endocytic sorting process causes dissociation of ligands from receptors. pH-sensitive binding has been reported previously for folate and vitamin $B_{12}$, both well-characterized ligands using RME mechanisms (Kamen and Capdevila, 1986).

In summary, our experiments demonstrate that a specialized, high-affinity riboflavin translocating system(s) exists in the intestinal cells with affinity values and biochemical characteristics similar to most receptor-mediated systems reported in the literature. This system is primarily expressed on the BL surface. Although the physiological role behind this type of polarity needs to be further investigated, our findings suggest that the BL uptake system could play an important role in supplying nutrients to the highly proliferative intestinal epithelium. These data aid in our understanding of the transepithelial transport mechanism of riboflavin and the proteins involved in its uptake and contribute to our knowledge of human riboflavin homeostasis and the physiology of B vitamins in general.

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**References**


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