CHARACTERIZATION OF THE ENDOCYTIC PATHWAYS REGULATING RIBOFLAVIN (VITAMIN B₂) ABSORPTION AND TRAFFICKING IN HUMAN EPITHELIAL CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Drug bioavailability is greatly compromised by the body’s innate defense mechanisms that result in low membrane permeability of hydrophilic drugs and macromolecules, reduced cellular retention by efflux transporters, and metabolic degradation by a multitude of enzymes. These factors, combined with underlying genetic aberrations and pathologies, drastically reduce drug bioavailability and, in certain instances, increase drug-related toxicity. To address many of these challenges, scientists have devised ways to target therapeutics into cells by coupling drugs directly (i.e., prodrug) or indirectly (e.g., polymer-mediated carriers) to natural ligands that are recognized by membrane receptors, and consequently gain cellular entry through receptor-mediated endocytosis (RME).

Although various receptors have been exploited to improve drug delivery, several receptor mechanisms remain to be defined. One RME target that has gained attention for its therapeutic potential in contraception, breast- and liver cancers involves the riboflavin (vitamin B₂) RME machinery. Our laboratory recently revealed riboflavin absorption to be regulated, in part, by clathrin-dependent RME (CME) in human epithelia. However, the identity of proteins involved in this process, and an understanding of the intracellular distribution of absorbed B₂ remain to be established.
The work presented in this dissertation addresses some of these areas of question using a three-tiered approach involving subcellular fractionation of $[^3H]$-B$_2$ dosed cells, 3D fluorescence colocalization analyses of rhodamine-labeled B$_2$ and immunostained endocytic markers, and RNA interference of the endocytic scission enzyme, dynamin 2 GTPase. Herein, we reveal dynamic enrichment of internalized riboflavin to CME endosomes, and to a lesser extent to the Golgi and mitochondria in human placental trophoblasts and enterocytes (Chapter 2). Furthermore, absorbed riboflavin is shown to be negatively coupled to cAMP levels. In Chapter 3, ~80% dynamin 2 protein knockdown causes ~40% reduced B$_2$-RME, and 2-fold higher B$_2$ localization at the plasma membrane of placental trophoblasts. Lastly, fluorescence colocalization assays exploring the involvement of clathrin-independent caveolae-mediated RME in B$_2$ internalization show minimal B$_2$ enrichment to caveolar endosomes (Chapter 4). Overall, our data demonstrate a dynamic subcellular B$_2$ trafficking itinerary largely associated with CME, and dynamin 2 is the first endocytic protein identified to regulate B$_2$ cellular entry in humans.
Dedicated to my parents, John R. and Cheryl K. Foraker, and my grandparents, John E. and Virginia E. Foraker, and Raymond H. and Mary K. Probasco
If I were asked to offer my perspective to those interested in pursuing their Ph.D. in biopharmaceutics or a related science, I would immediately inform them to be prepared to confront their own self-imposed barriers. Graduate school has been an odyssey of scientific and self discovery. I can’t emphasize enough the importance of networking with senior and junior colleagues throughout the graduate education process. I would like to offer my deepest thanks to my advisor, Dr. Peter W. Swaan, for instilling these very principles throughout my tenure in his lab. It has been through his constant guidance and support that has brought me to this honorable right of passage in my career development. A special thank you goes to my OSU advisor, Dr. Thomas D. Schmittgen, who provided me with the opportunity to broaden my research experience to include aptamer-based biomarker studies, and served as an invaluable resource in quantitative RT-PCR methods. I also want to express my gratitude to all the faculty of both The Ohio State University Graduate Program in Pharmaceutics and the Department of Pharmaceutical Sciences of the University of Maryland at Baltimore for fostering my graduate education. Especially, I would like to thank Drs. Kenneth K. Chan, James T. Dalton, Natalie D. Eddington, Hamid Ghandehari, William L. Hayton, Insong J. Lee, Robert J. Lee, and Paul S. Shapiro for their invaluable advice and support. If it had not
been for Dr. Kathleen Hillgren of Eli-Lilly & Co., I would not have been able to complete the last half of my research. Thank you, Kate, for your generosity in supporting my work. Also, I thank the laboratory of Dr. Mark A. McNiven (Mayo Clinic and Foundation, Rochester, Minnesota) for their generosity in donating validated dynamin 2 expression constructs, which were instrumental in corroborating our dynamin 2 RNA interference work.

I acknowledge the genuine friendship given to me from my colleagues, Dr. Se-Ne Huang, Dr. Yongheng Zhang, Dr. Cheng Chang, Dr. Mitch A. Phelps, Dr. Antara Banerjee, Dr. Vanessa M. D’Souza, Dr. Abhijit Ray, Dr. Julie A. Ray, Praveen M. Bahadduri, Chandra M. Khantwal, Clifford W. Mason, Lisa M. Bareford, Naissan Hussainzada, and Tatiana Claro Da Silva. It has been a privilege and honor to work with you all. I thank the administrative staff of both programs, with special gratitude to Colleen Day, Karen Lawler and Kathy Brooks for their generous support throughout my graduate studies. Also, I am forever indebted to Drs. Ana I. Robles and Wenqing Gao for their constant support, friendship and sincere words of encouragement during my graduate experience. I offer my sincere gratitude to Ms. Margaret MacLearie for generously offering her home as I was transitioning into graduate school and to Ms. Karen Sensabaugh for providing me with the knowledge and guidance to achieve balance amidst the many challenges that define graduate school.

I could not have made it through graduate school without my family of friends who openly took me into their hearts and enriched my life beyond the lab bench including, Kelly Baker, Christopher Bulka, Renee Cockerman, Veronica Cox, Cybele
Garcia, Devon Graham, Kathryn Kurre, and Alison Wright. I especially want to express my heartfelt gratitude to Dr. Andrew Hebbeler, who has acted as my steady compass during my time in Baltimore and constantly inspires me to be a better person. I can’t thank you enough for your friendship. A special thank you also goes to my steadfast and dear high school friend, Anne Householder, for being patient with me during the early years of my graduate studies and providing unconditional love and support throughout.

Mostly, I am grateful to my parents and grandparents, for their infinite words of wisdom and love, and for allowing me the priceless opportunities to follow my dreams. Thank you for believing in me. My deepest thanks also go to my sisters and brother-in-law, Matt and Jodi Toledo, and Lindsey Foraker for their constant love and support.
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<td>[ABC]-Transporter</td>
<td>ATP-Binding Cassette Transporter</td>
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<td>AP-2</td>
<td>Adaptor Protein 2</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>B$_2$</td>
<td>Riboflavin</td>
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<td>BCRP/ABCG2</td>
<td>Breast Cancer Resistance Protein</td>
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<td>BeWo</td>
<td>Human-Derived Placental Trophoblasts</td>
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<td>Br-cAMP</td>
<td>Membrane Permeable cAMP Analog</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>Ca$^{2+}$/Calmodulin-Mediated Pathway</td>
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<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<td>CAV1</td>
<td>Caveolin 1 Protein</td>
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<td>CCP</td>
<td>Clathrin-Coated Pit</td>
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<td>CME</td>
<td>Clathrin-Mediated Endocytosis</td>
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<td>CTMR4A</td>
<td>Carboxytetramethylrhodamine-4-Amine</td>
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<td>CTX</td>
<td>Cholera Toxin Subunit B</td>
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<td>CvME</td>
<td>Caveolae-Mediated Endocytosis</td>
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<td>DAPI</td>
<td>4', 6-Diamidino-2-Phenylindole, Dilactate</td>
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<td>EGTA</td>
<td>N'-Tetraacetic Acid</td>
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<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
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<td>FBP</td>
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<td>FMN</td>
<td>Flavin Mononucleotide</td>
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<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<td>GM130</td>
<td>130 kDa Membrane Associated Protein of the Golgi</td>
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<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<td>Guanosine Triphosphate</td>
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<td>GTPase</td>
<td>Enzyme that Hydrolyzes GTP</td>
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<td>H-89</td>
<td>Specific Protein Kinase A Inhibitor</td>
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<td>Hep G2</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HK-2</td>
<td>Human Renal Proximal Tubule Epithelia</td>
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<td>IBMX</td>
<td>3-Isobutyl-1-Methyl-Xanthine</td>
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<td>LAMP-1</td>
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<tr>
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<td>Post-Nuclear Supernatant</td>
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<td>Riboflavin Binding/Carrier Protein</td>
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RME  Receptor-Mediated Endocytosis
RNA  Ribonucleic Acid
RNAi  RNA Interference
SARA  Smad Anchor Protein for Receptor Activation
siRNA  Short-Interfering RNA
SITS  4-Acetamido-4'-Isothiocyanostilbene-2, 2'-Disulfonic Acid
Smad7-Smurf2  Protein Complex that Leads to Receptor Degradation
Smads  Cellular Cytoplasmic Gene Transcription Factors
SNX9  Sorting Nexin 9 Protein
TCA  Taurocholic Acid
TF  Transferrin, a Soluble Iron-Carrier Protein
TGF-b  Tumor Growth Factor Beta
TJ  Tight Junctions
VLDL  Very-Low-Density Lipoprotein
CHAPTER 1

INTRODUCTION

Riboflavin (also known as vitamin B₂) is a water-soluble vitamin essential for normal cellular functions, growth and development. In its coenzyme forms of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), B₂ performs key metabolic functions as an intermediary in the transfer of electrons in biological oxidation-reduction reactions. During periods of dietary deprivation or physiological and pathological stress, humans are vulnerable to developing B₂-deficiency. This may lead to a variety of clinical abnormalities, including growth retardation, anemia, skin lesions and degenerative changes in the nervous system (Cooperman, 1991). Humans cannot synthesize B₂ and, thus, must obtain the vitamin from their diet through absorption in the small intestine. Alternatively, riboflavin is also obtained from indigenous bacteria that colonize the large intestine, which naturally synthesize this vitamin. Although many studies have focused on the mechanism of B₂ uptake using various tissues and cell lines, transepithelial absorption is controversial and its exact mechanism(s) remains to be defined. Intracellular processes in B₂ absorption, such as cellular homoeostasis, and B₂
function and regulation are also poorly understood. The purpose of this chapter is to illustrate current in vitro and in vivo data defining B2 uptake mechanisms noted in different studies and to compare and contrast the various implicated parameters defining this (these) absorption mechanism(s). In addition, B2 subcellular trafficking events involving receptor-mediated endocytosis (RME) and cytoskeletal elements are discussed. The involvement of a soluble riboflavin binding/carrier protein (RfBP) in B2 uptake and trafficking in humans is currently elusive. Although several studies have reported its existence (Natraj et al., 1988; Prasad et al., 1992), it has not been characterized in detail. The majority of the work on RfBP has been focused on oviparous species and has shown a crucial role in embryo development. Few studies have been carried out on mammalian RfBP analogs despite its recent attention in therapeutic applications for contraception, and as a candidate biomarker of breast- and liver-cancer progression (Rao et al., 1999; Karande et al., 2001; Rao et al., 2006). This chapter further considers the similarities in protein sequence and function between RfBP for oviparous and mammalian species. Based on uptake and transport results using avian RfBP in the presence of B2, suggested roles in B2 absorption and trafficking events are presented. Since parallels can be drawn between the uptake and transport mechanisms of B2 and other well-studied vitamins, a brief discussion of commonalities defining such mechanisms for folate are illustrated. Here, the aim is to present comparisons with current knowledge on B2 uptake and transport, and how these prior studies may guide future elucidations of B2-specific pathways functioning in its homeostasis.
1.1 Clinical significance and models of riboflavin transport, storage and regulation

Flavoproteins constitute one of the largest groups of functionally related proteins currently known, catalyzing essential oxidation/reduction steps in almost every metabolic pathway in both prokaryotic and eukaryotic cells. Using essentially the same B2-based cofactor, flavoproteins participate in a remarkable array of biological processes, from simple electron transfer to complex signal transduction pathways. It is not surprising that defects within critical electron transfer processes may manifest themselves in severe, and sometimes fatal, metabolic diseases. Most dietary B2 is presented in the form of free riboflavin, FAD, FMN and flavoproteins, which must first be hydrolyzed to B2 before absorption can occur (Figure 1.1). Digestion of flavoproteins, FMN and FAD occurs by highly specific enzymes (Kasai et al., 1990), suggesting a physiologic preference that favors B2 as the absorptive species of dietary flavin. Though there is extensive information on the function of mucosal and cytosolic enzymes involved in the digestion of flavoproteins (e.g., flavokinase and FAD synthetase), it is interesting to note that none of these enzymes have been cloned or otherwise structurally characterized.

Therapeutically, B2 has recently gained renewed interest after it was shown to protect vital tissues from ischemia-induced oxidative injury resulting from heart attack or stroke (Hultquist, 1993). Given the critical role that B2 appears to play in pathological conditions with high morbidity, it is pertinent that its cellular homeostasis and the pathway(s) by which this vitamin gains entry into the body needs to be further defined.
Figure 1.1. Model illustrating B$_2$ absorption pathways and homeostasis along human intestinal epithelial cells. Following dietary intake and digestion (gastric acidification and proteases), B$_2$ analogs, FMN and FAD, are generated. These cofactors are further metabolized to the absorption active form, riboflavin, through the catalytic activities of various phosphatases. Extracellular levels of free B$_2$ may be bound by human RfBP prior to cellular uptake of the vitamin. At physiological concentrations, B$_2$ is taken into the cell through passive diffusion (A), receptor-mediated endocytosis (B), and/or a carrier-mediated mechanism (C). Once in the cell, B$_2$ is phosphorylated (Flavokinase) to FMN. FMN is either used as a prosthetic group of flavoenzymes, or converted to FAD through the action of FAD synthetase. FAD is commonly incorporated into flavoenzymes. Another possible fate of FMN and B$_2$ may involve transcytosis, where the receptor-bound B$_2$ or FMN complex is translocated to the basolateral membrane to allow release of the ligand in systemic circulation. Another possible role of a putative riboflavin-specific, basolaterally localized, receptor is to bind B$_2$ from the serum and translocate the vitamin into the cell to be used for various cellular maintenance and growth needs. Abbreviations: tight junctions (TJ), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD).
In animals, B₂-deficiency results in lack of growth, failure to thrive, and eventual death (Cooperman, 1991). Experimental riboflavin deficiency in dogs results in growth failure, weakness, ataxia, and inability to stand. The animals collapse, become comatose, and die (Cooperman, 1991). During the deficiency state, dermatitis develops together with hair-loss. Other signs include corneal opacity, lenticular cataracts, hemorrhagic adrenals, fatty degeneration of the kidney and liver, and inflammation of the mucus membrane of the gastrointestinal tract (Horwitt et al., 1972). Post-mortem studies in rhesus monkeys fed a B₂-deficient diet revealed that about one-third the normal amount of riboflavin was present in the liver (Foy and Mbaya, 1977), which is the main storage organ for B₂. These overt clinical signs of B₂-deficiency are rarely seen among inhabitants of developed countries. However, about 28 million Americans exhibit a common ‘sub-clinical’ stage (Lemoine et al., 1980), characterized by a change in biochemical indices (e.g., erythrocyte glutathione reductase, (Cooperman et al., 1973)). Although the effects of long-term sub-clinical B₂-deficiency are unknown, in children this deficiency results in stunted growth. Subclinical B₂-deficiency has also been observed in women taking oral contraceptives (Wynn, 1975), in the elderly, in individuals with eating disorders, and in disease states such as HIV (Beach et al., 1992), inflammatory bowel disease (Fernandez-Banares et al., 1989), diabetes and chronic heart disease (Cooperman, 1991). The fact that B₂-deficiency does not immediately lead to gross clinical manifestations indicates that the systemic levels of this essential vitamin are tightly regulated.
1.2 Riboflavin uptake mechanism(s)

Between the early 1940s and late 1970s, numerous studies described riboflavin pharmacokinetics and pharmacodynamics in man and other mammalian species. These initial studies provided the foundation of data suggesting the existence of a specialized B2 absorption mechanism (Jusko, 1975). Jusko and colleagues (Jusko et al., 1970) demonstrated in man a significant inhibition in renal clearance of B2 upon combined treatment with probenecid, now known to be a potent inhibitor of many organic anion transport systems. The same group reported pharmacokinetic data providing additional support for an active uptake mechanism. Using a three compartment open model, residual analysis of B2 renal clearance data in dog and man after rapid intravenous injections, 18.3 mg and 31 mg, respectively, revealed a saturable tubular reabsorption component (Jusko and Levy, 1970). Since the net renal clearance exceeded the glomerular filtration rate, B2 tubular secretion was also apparent. The maximal transport capacity was 33.3 µg/min for man, 21.9 µg/min. for dog, and the apparent K_m values were 16.3 and 9.61 µg/ml, respectively. Studies performed in the late 70s to early 1980s further suggested that riboflavin uptake and secretion involved the cyclic organic acid transport system. Jusko and Levy (Jusko, 1975) were the first to generate this hypothesis by showing the organic anion transport inhibitor, probenecid, to substantially block riboflavin transport in renal tubules. Additional support for this theory was presented by Spector (Spector, 1982), who showed saturable and energy-dependent B2 accumulation in rabbit kidney cortex slices. This accumulation was found to be inhibited by sulfhydryl reagents and cyclic organic acids such as 0.1 mM probenecid, which resulted in 37% inhibition compared to controls,
and no inhibition was observed in the presence of weak bases. Riboflavin was shown to be a competitive inhibitor of \( p \)-aminohippurate, a weak acid, and penicillin G uptake into kidney slices (Spector, 1982). Lowy and Spring (Lowy and Spring, 1990) showed similar effects of probenecid on \( B_2 \) transport into renal-derived MDCK cells. Using quantitative fluorescence video microscopy, they showed a 54% reduction in \( B_2 \) uptake in the presence of 1 mM probenecid. Increasing probenecid concentration to 10 mM resulted in near complete inhibition of \( B_2 \) transport. Combining the results of these studies illustrates that probenecid inhibits \( B_2 \) uptake in a dose-dependent manner in kidney cells and that \( B_2 \) absorption is suggested to involve a saturable, active uptake mechanism consisting of the carrier-mediated component, the organic anion transport system.

Upon oral administration of \( B_2 \), the fraction of the oral dose absorbed was approximately 50% (Jusko, 1975). Subsequent studies concentrated on the \( B_2 \) transport in isolated membrane vesicles or intact intestinal preparations (e.g., everted sacs and perfused segments) in an attempt to focus on the existence of a carrier-mediated component responsible for \( B_2 \) transport (Daniel et al., 1983; Said et al., 1985; Middleton, 1990; Said et al., 1993a; Said et al., 1993b). However, at the time of these publications, the cellular and molecular regulation of the intestinal uptake of \( B_2 \) was not clear and, thus, a definitive mechanistic conclusion could not be drawn. With the advent of well-characterized in vitro intestinal cell models, \( B_2 \) intestinal transport studies were better able to define kinetic transport parameters.
1.2.1 Saturable (active) transport component established in *in vitro* studies

Through the mid 1990s to the present, a vast amount of research has concentrated on defining the regulatory mechanisms involved in B₂ transport across different cell line models. Such studies have utilized cell systems representing colonic, intestinal, placental, renal and pulmonary epithelial cells, along with liver hepatocytes and peripheral blood mononuclear cells. However, kinetic parameters defining the maximal transport velocity and receptor/or transporter affinity constant appear to vary depending on the cell line or tissue being studied (Table 1.1). Reported \( K_m \) values range from low nM to low \( \mu \)M, and \( J_{\text{max}} \) values range from low fmol/mg of protein/min to low pmol/mg of protein/min (Kumar et al., 1998; Huang and Swaan, 2000; Said et al., 2000; Zempleni and Mock, 2000; Huang and Swaan, 2001). These discrepancies could be explained by the existence of multiple and distinct B₂ uptake mechanisms at varying levels of expression depending on cell lineage, or in part to the highly mutant prone characteristics defining the inherent nature of immortalized cell lines. Despite these differences, a trend is observed across different studies showing the B₂ uptake mechanism to be highly specific and saturable. B₂ competitive analog studies indicate the highest ligand affinity is continually expressed with unlabeled riboflavin that is in excess to basal concentrations of labeled riboflavin (Table 1.2). Half-maximal transport constants (comparable to Michaelis-Menten constants) range from 1.32 nM in placental trophoblast cells, BeWo (Huang and Swaan, 2001), to 0.67 \( \mu \)M in renal proximal tubule epithelial cells, HK-2 (Kumar et al., 1998). Riboflavin derivatives including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), lumiflavin, lumichrome, iso- B₂, and 8- \([\text{NH}_2]\)- B₂, show significant
inhibition in B₂ uptake as observed in competitive analog assays (Said and Ma, 1994; Kumar et al., 1998; Said et al., 2000; Zempleni and Mock, 2000; Huang and Swaan, 2001). Furthermore, these studies demonstrate the following analogs, listed in order of decreasing effects, exhibit marked inhibition in B₂ absorption: FMN (a phosphate group is attached to the alpha carbon of the ribityl side chain), FAD (with adenine diphosphate attached at the ribose moiety), lumiflavin (a methyl group replaces the ribose side chain), and lumichrome (completely lacks a ribose side chain) (Huang and Swaan, 2001).

Unrelated molecules such as biotin and mannitol, in addition to D-ribose and pterin ring-containing substances such as folate and pantothenic acid did not have any effect on B₂ uptake (Said and Ma, 1994; Kumar et al., 1998; Zempleni and Mock, 2000; Huang and Swaan, 2001). Combined, these data suggest the B₂ absorption mechanism does not require the ribose side chain. The highest substrate affinity constants observed by these effector analogs (i.e., those which lead to significant inhibition in B₂ uptake) were reported for the placental trophoblast cell model, BeWo (Huang and Swaan, 2001). The distinctively higher affinity in this cell line can be explained by clinical reports stating the crucial requirements that must be met during fetal nutrition and development (Zempleni et al., 1995).
Table 1.1. Summary of riboflavin uptake kinetics across divergent cell models. *All data presented was obtained under normal physiological conditions (i.e., basal concentrations for labeled riboflavin and the temperature was maintained at 37 °C throughout uptake experiments).*

1 Human-derived placental trophoblast cell line.
2 Human-derived colonic epithelial cell line.
3 Human-derived intestinal epithelial cell line.
4 Basolateral to apical flux (B→A); apical to basolateral flux (A→B).
5 Peripheral blood mononuclear cells (B cells, T cells and various granulocytes).
5b Proliferating peripheral blood mononuclear cells initially treated with a mitogenic agent, either concanavalin A (Con A) or pokeweed lectin (Lec).
6 Human-derived liver cell line.
7 Human-derived renal proximal tubule epithelial cell line.

<table>
<thead>
<tr>
<th>Cell Model</th>
<th>Riboflavin Concentration (nM)</th>
<th>$J_{\text{max}}$</th>
<th>$K_m$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BeWo²¹</td>
<td>1 to 1000</td>
<td>$286.83 \pm 28.89$ pmol/mg protein/20 min</td>
<td>$1.32 \pm 0.68$ nM</td>
<td>[i]</td>
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<tr>
<td>NCM460²²</td>
<td>5.5 to 1000</td>
<td>$3.29 \pm 0.58$ pmol/mg protein/3 min</td>
<td>$0.14 \pm 0.004$ µM</td>
<td>[ii]</td>
</tr>
<tr>
<td>Caco-2³⁸</td>
<td>0.5 to 160</td>
<td>$B \rightarrow A$: $188.20 \pm 0.88$ fmoles/min/cm²</td>
<td>$B \rightarrow A$: $4.06 \pm 0.03$ nM</td>
<td>[iii]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$A \rightarrow B$: $192.98 \pm 0.85$ fmoles/min/cm²</td>
<td>$A \rightarrow B$: $9.72 \pm 0.85$ nM</td>
<td></td>
</tr>
<tr>
<td>Caco-2</td>
<td>3.4 to 3000</td>
<td>$209.90 \pm 24.4$ pmol/mg protein/3 min</td>
<td>$0.3 \pm 0.03$ µM</td>
<td>[iv]</td>
</tr>
<tr>
<td>PBMC⁵⁵</td>
<td>5 to 1000</td>
<td>mean $J_{\text{max}}$: $38.5 \pm 20$ fmoles/10⁶ cells x 10 min</td>
<td>mean $K_m$: $955 \pm 344$ nM</td>
<td>[v]</td>
</tr>
<tr>
<td>PBMC⁵⁵</td>
<td>5 to 1000</td>
<td>Con $A$: $39 \pm 20$ fmoles/10⁶ x 10 min</td>
<td>Con $A$: $594 \pm 395$ nM</td>
<td>[v]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lec $A$: $23 \pm 10$ fmoles/10⁶ x 10 min</td>
<td>Lec: $513 \pm 470$ nM</td>
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<tr>
<td>Hep G2⁵⁰</td>
<td>33 to 3000</td>
<td>$3.56 \pm 0.24$ pmol/mg protein/3 min</td>
<td>$0.41 \pm 0.08$ µM</td>
<td>[vi]</td>
</tr>
<tr>
<td>HK-2⁴⁷</td>
<td>4.2 to 3000</td>
<td>$10.05 \pm 0.87$ pmol/mg protein/3 min</td>
<td>$0.67 \pm 0.21$ µM</td>
<td>[vii]</td>
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Table 1.2. Effects of related and un-related structural analogs on riboflavin uptake.

All competitive uptake assays were performed with physiologic concentrations of labeled $B_2$ in the presence or absence of excess structural analogs. All percentages represent the extent of labeled $B_2$ uptake inhibition as compared to respective untreated controls. Abbreviations are defined as follows: structural analogs were not tested in this study (nt); significant uptake inhibition was observed as compared with controls (+); an insignificant effect on $B_2$ uptake was reported (-); flavin mononucleotide (FMN); flavin adenine dinucleotide (FAD). $^1$Inhibitory constants were obtained using the Dixon Plot method. $^2$Significant uptake inhibition was observed with individual treatments of 8-$[\text{NH}_2]$-$B_2$ or iso-$B_2$. References: [i.] Huang, S.N. and Swaan, P.W. 2001; [ii.] Said, H.M. and Ortiz, A. et al. 2000; [iv.] Said, H.M. and Ma, T.Y. 1994; [v.] Zempleni, J. and Mock, D.M. 2000; [vi.] Said, H.M. and Ortiz, A. et al. 1998; [vii.] Kumar, C.K. and Yanagawa, N. 1998.
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<td>riboflavin</td>
<td>85%</td>
<td>nt</td>
<td>+</td>
<td>53 ± 22%</td>
<td>rt</td>
<td>+</td>
</tr>
<tr>
<td>FMN</td>
<td>73.50%</td>
<td>nt</td>
<td>nt</td>
<td>-</td>
<td>rt</td>
<td>rt</td>
</tr>
<tr>
<td>FAD</td>
<td>42.50%</td>
<td>nt</td>
<td>nt</td>
<td>-</td>
<td>rt</td>
<td>rt</td>
</tr>
<tr>
<td>lumiflavin</td>
<td>48%</td>
<td>+ K&lt;sub&gt;1&lt;/sub&gt; = 1.8 μM</td>
<td>+</td>
<td>nt</td>
<td>+ K&lt;sub&gt;1&lt;/sub&gt; = 1.84 μM</td>
<td>+</td>
</tr>
<tr>
<td>lumichrome</td>
<td>28.40%</td>
<td>+ K&lt;sub&gt;1&lt;/sub&gt; = 14.5 μM</td>
<td>+</td>
<td>38 ± 13%</td>
<td>+ K&lt;sub&gt;1&lt;/sub&gt; = 6.32 μM</td>
<td>+</td>
</tr>
<tr>
<td>8-[NH&lt;sub&gt;3&lt;/sub&gt;]-B&lt;sub&gt;2&lt;/sub&gt; or iso-B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>nt</td>
<td>nt</td>
<td>+ both&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-</td>
<td>rt</td>
<td>+ both&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>D-arabose</td>
<td>-</td>
<td>nt</td>
<td>-</td>
<td>-</td>
<td>rt</td>
<td>-</td>
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<tr>
<td>lumazine</td>
<td>nt</td>
<td>nt</td>
<td>-</td>
<td>nt</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pantothenic acid</td>
<td>nt</td>
<td>nt</td>
<td>-</td>
<td>nt</td>
<td>rt</td>
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<td>folic acid</td>
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<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>rt</td>
<td>nt</td>
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<td>amitriptyline</td>
<td>-</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>rt</td>
<td>nt</td>
</tr>
</tbody>
</table>
1.2.1.1. Common transport characteristics

Temperature-dependency of B₂ transport has been reported in different cell lines (Said and Ma, 1994; Zempleni and Mock, 2000; Huang and Swaan, 2001). In general, physiological temperatures (~ 37 °C) coincide with saturable uptake kinetics, whereas low temperature (~ 4 °C) correlates with linear absorption profiles (Said and Ma, 1994). This temperature-dependent regulation in vitamin absorption is a classic feature defining an active uptake mechanism. Nevertheless, this data alone does not distinguish between carrier- or receptor-mediated components regulating ligand translocation.

Metabolic and transport modulators are commonly implemented to define energy and membrane protein structural requirements that drive drug absorption. For the past decade in vitro systems have aided in the elucidation of the molecular and cellular events regulating B₂ absorption. As a result, we now have a better understanding of cellular regulatory events. For example, concerning energy-dependence, a regulatory role involving the Ca²⁺/calmodulin-mediated pathway (Ca²⁺/CaM), and a highly specific, saturable carrier-mediated component have been determined through competitive analog assays (Said and Ma, 1994; Kumar et al., 1998; Said et al., 1998; Zempleni and Mock, 2000; Huang and Swaan, 2001). Furthermore, the majority of studies suggest that B₂ absorption is Na⁺-independent.

In order to determine whether the absorption of B₂ is dependent on a motive energy force, metabolic and transport inhibitors have been employed, along with testing possible roles of ion-coupled gradients as analyzed through ion substitution assays (Tables 1.3 and 1.4).
Table 1.3. Effects of metabolic inhibitors on riboflavin transport across divergent cell models. The general effects of selected metabolic inhibitors on $B_2$ transport are indicated as significant in inhibiting uptake as compared to controls (+), insignificant or no observed alterations (−) or presented as the extent of uptake inhibition as compared to untreated controls (percentage). Abbreviations are defined as follows: metabolic inhibitor was not tested in study (nt); p-chloromercuriphenyl sulfonate (p-CMPS); p-chloromercuribenzenesulfonate (p-CMBS); 2, 4-dinitrophenol (DNP); and p-hydroxymercuriphenyl sulfonate (p-HMPS). \(^1\)Significant inhibition in labeled Rf uptake was noted at low temperatures (~ 4 °C). \(^2\)Uptake was performed using quiescent cells. \(^3\)The effects of changes in the osmolarity of the uptake medium on $B_2$ uptake were tested. \(^4\)Effects of 805 mosM media were assessed using quiescent and mitogen-stimulated PBMC, respectively, and increased $B_2$ uptake into proliferating PBMC were correlated with increased cell volume due to blastogenesis. References: [i.] Huang, S.N. and Swaan, P.W. 2001; [ii.] Said, H.M. and Oritz, A. et al. 2000; [iv.] Said, H.M. and Ma, T.Y. 1994; [v.] Zempleni, J. and Mock, D.M. 2000; [vi.] Said, H.M. and Oritz, A. et al. 1998; [vii.] Kumar, C.K. and Yanagawa, N. 1998.
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<tr>
<td>Temperature$^1$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+$^2$</td>
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<td>+</td>
</tr>
<tr>
<td>Osmolality$^3$</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>38 to 55%$^4$</td>
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**Metabolic Inhibitors**

<table>
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<tr>
<th></th>
<th>BeWo [Ⅰ]</th>
<th>NCM460 [Ⅰ]</th>
<th>Caco-2 [Ⅰ]</th>
<th>FBMC [Ⅰ]</th>
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<tr>
<td>Sodium azide</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nt</td>
</tr>
<tr>
<td>2-deoxyglucose</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>ρ-CMPS</td>
<td>nt</td>
<td>+</td>
<td>nt</td>
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<td>ρ-CMBS</td>
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<td>DNP</td>
<td>nt</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td>ρ-HMPS</td>
<td>nt</td>
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</table>
Table 1.4. Effects of ion substitutions, organic anions, anion exchangers or organic anion transport inhibitors on $B_2$ transport. All percentages represent the extent of $B_2$ uptake inhibition as compared to controls unless stated otherwise.  

1 Ionic coupling properties suggested for $B_2$ transport were tested through ion substitution assays, and is indicated as $X$ ion isosmotically replaces $Y$ ion ($X \rightarrow Y$).  

2 Significant inhibition on $B_2$ uptake was exclusively observed after prolonged incubation in Na$^+$-depleted conditions.  

3 Effect is indicated as the percentage of inhibition in $B_2$ uptake as compared to controls.  

4 An increase in $B_2$ uptake was noted as pH changed from pH 8 to 7.  

5 A gradual decrease in $B_2$ uptake was observed as pH was changed from pH 8 to 5.  

6 Inhibition constant ($K_i$) was determined to be 0.86 mM as determined by the Dixon method.  

7 Inhibition constant ($K_i$) was determined to be 0.48 mM as determined by the Dixon method. Abbreviations and symbols are defined as follows: significant inhibition in $B_2$ uptake (+); no effect on $B_2$ uptake observed (-); treatment was not tested in study (nt); 4,4’-diisothiocyanostilbene-2,2’disulfonic acid (DIDS); 4-acetamido-4’-isothiocyanostilbene-2,2’-disulfonic acid (SITS); ethylene glycol-bis(beta-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA). References: [i.] Huang, S.N. and Swaan, P.W. 2001; [ii.] Said, H.M. and Ortiz, A. et al. 2000; [iv.] Said, H.M. and Ma, T.Y. 1994; [v.] Zempleni, J. and Mock, D.M. 2000; [vi.] Said, H.M. and Ortiz, A. et al. 1998; [vii.] Kumar, C.K. and Yanagawa, N. 1998.
### Cell Model Systems

<table>
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<tr>
<th>$\text{E}_2 \text{ Uptake Modulators}$</th>
<th>BeWo [L]</th>
<th>NCM460 [u.]</th>
<th>Caco-2 [iv.]</th>
<th>PBMC [v.]</th>
<th>Hep G2 [vi.]</th>
<th>HK-2 [vi.]</th>
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<tr>
<td>Ion-Coupling Properties$^3$</td>
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<tr>
<td>$\text{Cl} \rightarrow \text{Cl}$</td>
<td>71.49 ± 6.18%</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
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<tr>
<td>Iodoacetate $\rightarrow \text{Cr}$</td>
<td>66.85 ± 5.09%</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
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<tr>
<td>gluconate $\rightarrow \text{Cr}$</td>
<td>84.39 ± 5.23%</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
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<tr>
<td>Chloride $\rightarrow \text{Na}^{+}$</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
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<td>$\times$</td>
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<tr>
<td>Ouabain</td>
<td>$\times$</td>
<td>$\times$</td>
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</tr>
<tr>
<td>$\text{K}^{+} \rightarrow \text{Na}^{+}$</td>
<td>$\times$</td>
<td>$\times$</td>
<td>$\times$</td>
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<tr>
<td>$\text{Li}^{+} \rightarrow \text{Na}^{+}$</td>
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$^3$ Organic Anions, Anion Exchanger and Organic Anion Transport Inhibitors

| Probenecid   | -   | -   | $\times$ | $\times$ | $\times$ | +   |
| Furosemide   | -   | -   | $\times$ | $\times$ | $\times$ | $\times$ |
| DIDS         | average 75% | - | $\times$ | $\times$ | $\times$ | +   |
| STS          | $\times$ | $\times$ | $\times$ | $\times$ | $\times$ | +   |
| $p$-aminophenylurate | $\times$ | $\times$ | $\times$ | $\times$ | $\times$ | +   |
| penicillin G  | $\times$ | $\times$ | $\times$ | $\times$ | $\times$ | +   |
| salicylate    | $\times$ | $\times$ | $\times$ | $\times$ | $\times$ | +   |
Consistent effects leading to marked inhibition in B₂ uptake, though varying on the extent of inhibition between different cell lines, are seen with sodium azide treatments (Said and Ma, 1994; Kumar et al., 1998; Said et al., 1998; Said et al., 2000; Huang and Swaan, 2001). Sulfhydryl group modifying agents such as p-chloromercuriphenyl sulfonate (p-CMPS), p-chloromercuribenzenesulfonate (p-CMBS), iodoacetate, and eosin maleimide, cause significant inhibition in B₂ uptake in colon, intestine, liver, and renal proximal tubule cell systems (Said and Ma, 1994; Kumar et al., 1998; Said et al., 1998; Said et al., 2000). These results, which are in agreement with earlier pharmacokinetic/pharmacodynamic and ex vivo tissue studies (Jusko and Levy, 1970; Jusko, 1975; Spector, 1982), suggest a possible role for reducing equivalents (e.g., NADH and FADH₂) in the absorption process. Added support for this theory was demonstrated in p-CMBS-pretreated Caco-2 cell monolayers that were subsequently exposed to reducing agents, dithiothreitol or mercaptoethanol, which were found to reverse the effects of the sulfhydryl agent (Said and Ma, 1994). Energy in the form of ATP is also suggested to be involved in B₂ uptake. Said and coworkers (Said and Ma, 1994) report significant (P < 0.01) inhibition in B₂ (3.4 nM) uptake across Caco-2 cell monolayers when treated with 2,4-dinitrophenol (1 mM), which is known to reduce intracellular ATP. However, B₂ transport appears to not be directly coupled to ATP sources.

Transporters are generally fueled through the cotransport of ions (solute transport family) or through a coupled catabolic reaction involving adenosine triphosphate (ATP) hydrolysis (ATP binding cassette [ABC]-transport family). However, Na⁺ or H⁺
mediated-cotransport of organic solutes is more frequently observed in mammalian species (Hediger et al., 1995). The role of Na\(^+\) in B\(_2\) uptake has been controversial. In general, a sodium-independent process has been observed across inherently diverse cell lines (Table 1.4). Yet, prior to the early 1990s, several studies utilizing intestinal tissue preparations reported B\(_2\) absorption to be dependent on Na\(^+\) (Daniel et al., 1983; Middleton, 1985; Said and Arianas, 1991). Said and Ma demonstrated a general Na\(^+\)-independence in B\(_2\) uptake studies using Caco-2 cell monolayers (Said and Ma, 1994). Their studies involved replacing Na\(^+\) ions with choline, Li\(^+\), mannitol, or K\(^+\) (Table 1.4). All replacement conditions showed no effect on B\(_2\) transport, except for K\(^+\), which led to 24% (P < 0.01) inhibition as compared to controls. In addition, with prolonged incubation periods under Na\(^+\)-depleted conditions, the K\(^+\) ion inhibiting effects markedly increased to 42%. This reduction in uptake has been suggested to be a result of depolarizing effects that K\(^+\) ions or Na\(^+\)-depleted growth conditions can have on inherent biochemical maintenance processes in cells and in intact tissue preparations (Said and Ma, 1994). Additional support for Na\(^+\)-independence has been shown through uptake studies performed in the presence of ouabain, a well known Na\(^+\), K\(^+\)-ATPase inhibitor. Such studies repeatedly reveal no significant effects on B\(_2\) uptake with this agent (Kumar et al., 1998; Said et al., 1998; Said et al., 2000; Zempleni and Mock, 2000; Huang and Swaan, 2001).

In general, B\(_2\) uptake appears independent of environmental pH (Table 1.4). However, Said and coworkers revealed a slight increase in B\(_2\) uptake with media pH near the physiologic range (~ pH 7), whereas, more acidic conditions led to a gradual decrease
in uptake (Said et al., 1998; Said et al., 2000). Such pH-coupled effects may be explained through a putative ligand-receptor uncoupling event that is common in receptor-mediated endocytosis. This uncoupling reaction, which is known to take place shortly after receptor-ligand endocytosis, is accompanied by a reduction in endosomal pH (~ pH 5) (Mukherjee et al., 1997; Swaan, 1998). However, reduced pH in the extracellular media can theoretically mimic the intracellular effects of decoupling the ligand from its receptor. Our laboratory reported a pH-dependent dissociation of surface bound B₂ in human intestinal cells (Caco-2) (Huang and Swaan, 2000). A further implication of a receptor-mediated component in B₂ uptake is discussed in later sections of this chapter.

A recent study by our laboratory suggests the involvement of Cl⁻ ions in B₂ uptake as tested in placental BeWo cells (Huang and Swaan, 2001). Isoosmotic replacement of Cl⁻ ions with iodide, isocyanate, or gluconate significantly inhibited B₂ uptake as compared to controls (Table 1.4). Chloride ion effects have not been thoroughly studied in other cell lines, and thus additional investigations are warranted.

As indicated in the early studies by Levy and colleagues (Jusko et al., 1970), B₂ uptake was significantly reduced in the presence of the organic anion transport inhibitor, probenecid. These results along with additional studies on renal tissue further suggested B₂ absorption was regulated through the organic anion transport system (Jusko, 1975; Spector, 1982; Lowy and Spring, 1990). As a result several subsequent studies have tested whether probenecid along with furosemide (Na⁺, K⁺-2Cl⁻-cotransporter inhibitor), 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (also known as DIDS, a membrane impermeable anion-exchanger/chloride channel inhibitor), 4-acetamido-4’-
isothiocyanostilbene-2,2’-disulfonic acid (also known as SITS, an organic anion transport inhibitor), and/or various organic anions (e.g., p-aminohippurate, penicillin G and salicylate) generate similar inhibition effects on B₂ absorption in non-renal cells (Table 1.4). Interestingly, excluding DIDS, none of these agents resulted in any significant inhibition. However, as shown by Huang and coworkers (Huang and Swaan, 2001), DIDS (for all tested concentrations 0.1, 0.5, and 1.0 mM) caused an average of 75% reduction in B₂ uptake in BeWo cells. Such results further corroborate the putative role of Cl⁻ ions in cellular riboflavin homeostasis in placental trophoblasts. These studies do not indicate whether Cl⁻ ion concentrations are directly or indirectly involved in the translocation of B₂ across a plasma membrane, and thus additional studies are warranted. Nonetheless, the general inhibitory effect of organic anion modulators on B₂ uptake appears to be exclusive to renal proximal tubule cells (Kumar et al., 1998).

Cellular regulation of B₂ absorption, particularly cyclic nucleotide-dependent pathways, has been investigated using such modulators as 3-isobutyl-1-methyl-xanthine (IBMX, a protein kinase A (PKA)-activator), H-89 (specific PKA inhibitor), pCPT-cGMP (membrane permeable analog of cGMP), forskolin (adenylyl cyclase activator), Br-cAMP (membrane permeable cAMP analog), cholera toxin (increases intracellular cAMP levels), and dibutryl cAMP (also increases intracellular cAMP) (Table 1.5). Huang and coworkers demonstrated an immediate and significant reduction in B₂ uptake upon short term incubation with IBMX using BeWo cells (Huang and Swaan, 2001). This effect was accompanied by marked changes in K_m and J_max. In a similar fashion, the cGMP analog, pCPT-cGMP, significantly inhibited B₂ absorption (Huang and Swaan,
2001), which suggests a possible role for the protein kinase G (PKG) pathway. In addition, coincubating with both modulators resulted in a potentiated uptake reduction (reduced uptake fell to 43.7% and 15.3% as compared to individual treatments with pCPT-cGMP or IBMX, respectively). Similar studies in Caco-2 cells with IBMX induced significant uptake inhibition in a concentration dependent manner (Said and Ma, 1994). Overall, data suggest a possible role for the PKA and/or PKG pathways in riboflavin absorption.
Table 1.5. Effects of various modulators targeting cAMP, protein tyrosine kinase, protein kinase A, G, C and calmodulin-mediated pathways on $B_2$ transport. $^1$Effect of these modulators was exclusively observed for differentiated, syncytiotrophoblast, cells. $^2$Combination treatment significantly enhanced inhibiting effects on $B_2$ uptake. $^3$The inactive analog of bisindolylmaleimide I, bisindolylmaleimide V, also led to same degree of inhibition on $B_2$ uptake, and thus effects were nonspecific. $^4$Slight insignificant inhibition in $B_2$ uptake was observed, however, inactive analogs of indicated agents resulted in similar results, thus effects were nonspecific. Abbreviations and symbols are defined as follows: significant inhibition in $B_2$ uptake (+); no effect on $B_2$ uptake observed (-); treatment was not tested in study (nt); treatment led to concentration dependent inhibition in $B_2$ uptake (CD); treatment led to significant increase in $K_m$ ($K$) and/or significant decrease in $J_{max}$ ($J$). References: [i.] Huang, S.N. and Swaan, P.W. 2001; [ii.] Said, H.M. and Ortiz, A. et al. 2000; [iv.] Said, H.M. and Ma, T.Y. 1994; [vi.] Said, H.M. and Ortiz, A. et al. 1998; [vii.] Kumar, C.K. and Yanagawa, N. 1998.
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**Modulators of Protein Kinase C, Protein Tyrosine Kinase, or Calmodulin-Mediated (CaM) Pathways**

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The influence of the protein kinase C (PKC) pathway was investigated using phorbol-12-myristate-13-acetate (PMA, a PKC activator), chelerythrine (specific PKC inhibitor), or staurosporin (also a PKC inhibitor). However, data clearly indicate that this pathway is not involved in B₂ uptake (Table 1.5) (Said and Ma, 1994; Kumar et al., 1998; Said et al., 1998; Said et al., 2000; Huang and Swaan, 2001).

The involvement of Ca²⁺/CaM in B₂ absorption has been established through the use of various modulators that are specific to this pathway. Of these modulators, calmidazolium, a Ca²⁺/CaM antagonist, has been commonly implemented. At the molecular level, calmidazolium has been shown to activate phospholipases C and A₂ that potentiate the release of cellular Ca²⁺ stores and also Ca²⁺ entry across a variety of mammalian cell systems (Peppiatt et al., 2004). Interestingly, several studies report a significant inhibition in B₂ uptake by calmidazolium treatment in three distinct cell models, BeWo (human-derived placental trophoblasts), NCM460 (human-derived colonic epithelial cell line), and Hep G2 (human-derived liver cells) (Said et al., 1998; Said et al., 2000; Huang and Swaan, 2001). Furthermore, these reports demonstrate that calmidazolium inhibits B₂ uptake in a concentration dependent manner (Table 1.5). Said and coworkers further demonstrated a marked reduction in the maximal transport velocity ($I_{max} = 0.135 \pm 0.003$ and $0.084 \pm 0.007$ pmol/mg protein/3 min for control and 10 μM calmidazolium, respectively ($P < 0.01$)) in colonic epithelial cells (Said et al., 2000). This effect was accompanied by a significant increase in the apparent $K_m$. Similar effects were noted with calmidazolium treatments in Hep G2 (Said et al., 1998) and HK-2 cells (Kumar et al., 1998). Other Ca²⁺/CaM modulators tested have included W-13 (a specific
Ca\textsuperscript{2+}/CaM antagonist that is structurally different from calmidazolium), KN-93 (a selective Ca\textsuperscript{2+}/CaM-dependent kinase II inhibitor), trifluoperazine (a Ca\textsuperscript{2+}/CaM inhibitor), and KN-62 (a Ca\textsuperscript{2+}/CaM inhibitor) (Kumar et al., 1998; Said et al., 1998; Said et al., 2000; Huang and Swaan, 2001). All inhibitors, excluding KN-93, led to a significant reduction in B\textsubscript{2} uptake. The largely consistent effect of these Ca\textsuperscript{2+}/CaM modulators on riboflavin homeostasis as observed across divergent human cell models suggests the molecular mechanism(s) regulating the cellular entry of this vitamin are sensitive to Ca\textsuperscript{2+} levels.

1.2.2. Passive diffusion component and an adaptive regulatory mechanism in B\textsubscript{2} uptake

Along with indications of a B\textsubscript{2} specific, active transport process, a passive or simple diffusion component has been observed in cell culture under certain growth conditions. At riboflavin concentrations higher than in human plasma (>12nM), Huang and colleagues reported B\textsubscript{2} absorption in intestinal epithelial cells, Caco-2, to predominantly exhibit passive diffusion kinetics, whereas, saturation kinetics are observed at near basal B\textsubscript{2} concentrations (≤12 nM for humans). The conditions favoring this passive component could be further explained by reports indicating an adaptive regulatory absorption mechanism as evidenced in B\textsubscript{2}-deficient and over supplemented growth conditions. This adaptive phenomenon has been observed in renal (Kumar et al., 1998), colon (Said et al., 2000), and intestinal (Said and Ma, 1994) epithelial cells. Intestinal cells initially grown in B\textsubscript{2}-deficient media and subsequently exposed to physiological B\textsubscript{2} concentrations exhibit a marked increase in riboflavin uptake compared to controls (Said and Ma, 1994). Conversely, over supplemented B\textsubscript{2} conditions led to a
marked reduction in $J_{\text{max}}$ (129 ± 13 pmol/mg protein/3 min). However, the apparent $K_m$ values did not change in both cases (Said and Ma, 1994). Similar results were obtained in colon epithelial cells (Said et al., 2000). Renal proximal tubule cells, HK-2 (Kumar et al., 1998), revealed similar changes in maximal transport velocity under deficient and over supplemented growth conditions. However, the apparent $K_m$ values were inconsistent: $J_{\text{max}}$ increased in the B2-deficient state, whereas the corresponding apparent $K_m$ value significantly decreased (69% of control); $J_{\text{max}}$ decreased under over supplemented conditions, whereas the corresponding $K_m$ increased (127% of control). Overall, these findings suggest the existence of multiple uptake mechanisms in renal cells that are differentially regulated by varying levels of B2. Effects to the maximal transport velocity in all three models suggest that expression or activity of the proteins involved in B2 uptake are correlated with changes in B2 concentration. Said and coworkers further questioned whether such adaptive uptake responses were attributed to transcriptional events (Said et al., 2000). Upon treating NCM460 cells with a transcription inhibitor, actinomycin D, 24 hours after cells were maintained in either B2-deficient or over supplemented conditions, B2 uptake was unaffected in the over supplemented situation, but, significant inhibition was noted for B2-starved cells. This latter finding suggests transcriptional events may function in up-regulating protein machinery necessary for efficient B2 absorption during periods of vitamin deficiency. At the other extreme, i.e., B2-over supplemented conditions, protein machinery are suggested to be down regulated and may explain why a passive diffusion component dominates at these higher riboflavin concentrations.
1.3. Receptor-mediated and/or carrier-mediated transport?

Until recently, it was generally accepted that B2 translocation followed an active, carrier-mediated pathway. However, the criteria used to define active transport, i.e. energy dependency and saturation transport kinetics, do not effectively distinguish carrier-mediated transport from receptor mediated endocytosis. Receptor-mediated events involve endocytosis followed by microtubule driven vesicular sorting to various cellular organelles (Mukherjee et al., 1997). In an effort to determine whether a receptor mediated component is involved in B2 transport, our laboratory treated Caco-2 cells with either brefeldin A (BFA), which induces misrouting of vesicles at the trans-Golgi network, or nocodazole, a microtubule depolymerizing agent (Huang and Swaan, 2000). Our data revealed nocodazole significantly inhibited the basolateral to apical flux of riboflavin as well as fluorescently labeled transferrin (56.7% and 31.8% of control, respectively), an iron transport protein that has been extensively established to be internalized by a transferrin-specific receptor via receptor-mediated endocytosis. In addition, the apical to basolateral transport of both riboflavin and transferrin was significantly increased (37.1% increase compared to controls for riboflavin) upon treatment with nocodazole. BFA led to a significant increase in transport in both directions for transferrin (13-fold higher in apical to basolateral, and 5-fold increase in basolateral to apical). BFA caused a slight, though not significant, increase in basolateral to apical B2 transport, and a significant increase was observed for the apical to basolateral flux. The effects of BFA on inducing increased concentrations of membrane receptor on the apical membrane as opposed to the
basolateral membrane are comparable to similar studies involving transferrin transport by Shah and Shen (Shah and Shen, 1994).

More recently, the subcellular localization of B2 in BeWo cells was studied (Huang et al., 2003). Rhodamine-labeled riboflavin (Rd-RF) and FITC-labeled transferrin (FITC-Tf) were used to trace subcellular distribution patterns of both compounds upon cellular uptake. Specifically, internalized fluorescently-labeled ligands were examined for 2D colocalized signals with indirect-immunostained RME markers for human clathrin heavy chain and LAMP-1, a lysosome-associated membrane protein. Using fluorescence microscopy, a distinct perinuclear punctate staining pattern was observed for rhodamine-B2, which colocalized with clathrin and FITC-Tf. Furthermore, a membrane diffusible probe specific for acidic organelles (e.g. late endosomes and lysosomes), LysoTracker Blue-white DPX, did colocalize with Rhodamine-B2. Colocalization of riboflavin was also observed with Rab5, a small GTPase that specifically resides with early endosomes, and LAMP-1. All these findings suggest B2 transport involves clathrin dependent RME in BeWo cells (Huang et al., 2003).

1.4. Riboflavin binding/carrier protein (RfBP)

Plasma protein binding and translocation of riboflavin in mammals are non-specific under normal conditions. During pregnancy, this non-specific transport by albumin and riboflavin binding immunoglobulins is not adequate to provide the developing fetus with sufficient B2 for normal development and growth. It is believed that under these demanding conditions specific carrier proteins are expressed that deliver riboflavin to the developing embryo. These soluble plasma proteins are referred to as
“riboflavin binding proteins” (RfBP) or “riboflavin carrier protein” (RCP). It is important to clarify that RfBP, originally discovered in chicken, is not a membrane spanning carrier protein but a soluble protein that delivers B<sub>2</sub> to the cell possibly by a receptor-mediated mechanism. Pregnancy-specific RfBP has been found in rat (Muniyappa and Adiga, 1980), mouse (Natraj et al., 1987), bovine (Merrill et al., 1979), simian (Visweswariah and Adiga, 1987b), spadefoot toad (Storey et al., 1999), turtle (Hamajima and Ono, 1995) and human plasma (Visweswariah and Adiga, 1987a). Though the mechanism of the transport process remains elusive, it is believed that more then one mode of transport process may exist, which involves a receptor and/or carrier mediated transport mechanism as reported for the transport of other micronutrients, such as folic acid.

1.4.1 Functions in oviparous species

Chicken RfBP is the first riboflavin specific carrier to be identified, and thus the most extensively studied. It was originally isolated from egg yolk (Rhodes et al., 1959), but later it was found that it is also present in egg white (Ostrowski et al., 1968) and plasma of egg laying hens (Miller et al., 1982). RfBP in egg constitutes 0.8% (Rhodes et al., 1959) of the egg white proteins present, which is relatively abundant amongst egg white proteins. There are three known forms of RfBP that are the product of the same gene and undergo different post-translational modifications (Hamazume et al., 1984; Hamazume et al., 1987; Rohrer and White, 1992; Tarutani et al., 1993). The RfBP gene is expressed both in liver and the oviduct of egg laying hens (Zheng et al., 1988). Despite reports of RfBP in other birds and reptilian sources, such as the Japanese quail (Walker et al., 1991), Indian python, painted turtle and American alligator (Abrams et al., 1988),
none of the amino acid sequences have been reported. So far, among the reptilian species the turtle, *Pelodiscus sinensis*, gene sequence is exclusively known (Hamajima and Ono, 1995). In the amphibious spade foot toad, *Scaphiopus couchii*, RfBP is among a group of genes that are induced or up-regulated during estivation (Storey et al., 1999).

In chicken, it is now well established that B$_2$ is transported to the egg by the egg laying hen with the help of RfBP. Chicken RfBP (cRfBP), a 37 kDa protein, is one of the best characterized riboflavin binding proteins. Egg white RfBP is 35% saturated with riboflavin as compared to fully saturated yolk RfBP. It is believed that RfBP in egg white serves as the scavenger of free riboflavin, which may protect egg white from bacterial attack (Tranter and Board, 1982).

In *Scaphiopus* toads the need for micronutrients during breeding is provided by an increased expression of RfBP along with other micronutrient carrier proteins (Storey et al., 1999). The developing embryo has higher nutrient demands, and most of the proteins produced are under estrogen control. Estrogen-dependent expression of cRfBP is distinct from that of other egg proteins such as egg white ovalbumin which is synthesized in the oviduct, or vitellogenin which is synthesized in the liver. Chicken RfBP is synthesized in both the liver and oviduct (Norioka et al., 1985).

1.4.1.1 Interspecies sequence homology and identity

Avian plasma RfBP is produced in the liver under estrogen control and includes a 17 amino acid signal peptide that is cleaved post-translationally. Egg white and yolk RfBP are formed as a result of proteolytic cleavage of the last 11-13 amino acids of plasma RfBP, and takes place when the molecule crosses the vitellogen membrane of the
oocyte (Norioka et al., 1985). In an estrogen induced turtle it has been reported that unlike chicken RfBP, the turtle RfBP gene contains an open reading frame of 242 amino acids with a signal peptide of 18 amino acids (Hamajima and Ono, 1995). Hamajima and coworkers found overall 71.3% amino acid identity between the deduced amino acid sequences of turtle and chicken RfBP. In a separate study, Storey and coworkers (Storey et al., 1999) found that spade foot toad RfBP has ~ 50% sequence identity with chicken (49.6%) and turtle (50.4%) RfBP. The area of greatest species variation between the three proteins was within the 17-18 residue signal sequence and the final 25 residues near the C-terminus. Furthermore, of the tissues examined, RfBP was shown to be largely liver specific, as no RfBP mRNA was detected in brain, gut, heart or kidney.

1.4.1.2 Suggested mechanism of RfBP in riboflavin homeostasis

As mentioned earlier, RfBP has been found to be essential for the developing embryo in chicken. Birds with a genetic defect in the RfBP gene lay fertilized eggs normally, but their eggs fail to hatch due to a lack of riboflavin disposition (MacLachlan et al., 1993). Chicken RfBP in oocytes binds to lipid carrier vitellogenin in a 1:1 molar ratio, and the macromolecular complex of the two carriers recognizes a multifunctional oocyte specific lipoprotein receptor (Mac Lachlan et al., 1994). The binding to vitellogenin is thought to be through a highly phosphorylated region on cRfBP that extends from amino acids 186-197, containing 8 serine-bound phosphate residues. Removal of any phosphate residue from RfBP reduces oocyte uptake in vitro by over 60% (Miller et al., 1982). This macromolecular complex binds to an oocyte specific LDL receptor that can also bind to low-density lipoprotein (LDL), very low-density lipoprotein
(VLDL) and vitellogenin independently (Sooryanarayana et al., 1998). The interaction between vitellogenin and RfBP is thought to be through phosphate-calcium-phosphate bridges involving phosphorylated residues in both proteins, as the interaction was reported to be optimum in the presence of free phosphate and calcium (Mac Lachlan et al., 1994). The authors in the study pointed out the differences in the molar concentrations of vitellogenin (six-fold) and RfBP (nine-fold), which does not correspond with a serum 1:1 molar ratio and suggests that there may be additional mechanisms of RfBP uptake by the oocyte. Recent studies have indicated the phospho-peptide of RfBP may play an important role in the binding of this protein to its membrane receptor (Sooryanarayana et al., 1998). This is supported by data that revealed vitellogenin inhibits RfBP binding to its specific protein. Since vitellogenin binds to receptors of the low-density lipoprotein receptor family and LDL receptors, which can interact with polyanionic ligands, it was predicted that the family of LDL receptors and LDL related proteins are potential candidates for such interactions. To date this relationship remains to be defined.

1.4.2 Mammalian RfBP

In rats, Sertoli cells maintain the tubular biochemical microenvironment for germ cell proliferation and differentiation in the lumen of seminiferous tubules through selective transport and polarized secretion. They also act as a blood testis barrier to provide a serum free microenvironment. Nearly all major carrier proteins are produced in Sertoli cells as there is a higher nutrient demand in the differentiating cells (Skinner and Griswold, 1980; Skinner and Griswold, 1983; Collard et al., 1988; Davis and Ong, 1992). Since follicle stimulating hormone (FSH) and testosterone increase the synthesis of both
cellular and secreted proteins in Sertoli cells, it was predicted that a riboflavin binding protein also exists in these cells (Subramanian and Adiga, 1996a). A protein species that was synthesized and secreted by immature rat Sertoli cells was shown to be similar to chicken RfBP at the biochemical and immunological levels (Subramanian and Adiga, 1996b). To understand the importance of RfBP in embryonic development, estrogenic modulation of RfBP gene expression has been tested in subhuman primates (bonnet monkeys) through immunochemical studies (Visweswariah and Adiga, 1988). It was observed that serum RfBP levels increased with enhanced estradiol secretion during the menstrual cycle and early pregnancy. In humans, the existence and role of RfBP is more elusive. Human cord serum contains proteins capable of binding riboflavin (Natraj et al., 1988). Furthermore, reports have shown rodent-derived and non-human primate RfBP cross-react with anti-chicken antibodies (Seshagiri and Adiga, 1987; Subramanian and Adiga, 1996b). Hence it was suggested that the transport of riboflavin to the developing mammalian embryo could be a carrier protein-mediated process analogous to that of riboflavin disposition in the chicken oocyte. A recent study by our laboratory revealed a 95% reduction in riboflavin absorption in human placental trophoblasts (BeWo) that were treated with an anti-chicken RfBP antibody (Mason et al., 2006). In addition, this same report showed both $[^3\text{H}]-\text{B}_2$ and $^{125}\text{I}$-RfBP to be subcellularly enriched to endosomal and lysosomal organelles isolated by sucrose-density fractionation. Similar to avian species, these results may suggest that a B$_2$-specific soluble carrier protein is important in mediating this vitamin’s cellular absorption in human placental trophoblasts. Another interpretation of this data may be that the antibody to the chicken RfBP is merely
expressing an epitope that shares biochemical conformational similarities to the elusive riboflavin receptor or a binding protein expressed in humans. However, the evidence of a soluble RfBP analog with biochemical and immunological similarities to avian RfBP reported for several divergent mammalian species (e.g., rat, bovine, and simian) suggest this secreted protein is evolutionarily conserved, and further strengthens the hypothesis that such a B₂-sequestering protein potentially exists in humans.

1.4.2.1 Putative function and role of RfBP in riboflavin uptake and transport

Immunohistochemical localization revealed that RfBP exists in rodent Sertoli cells, Leydig cells, pachytene spermatocytes, around spermatids and on mature spermatoza of rodents and primates (Bhat et al., 1995). A three-fold increase in RfBP levels in Sertoli cells was reported to correspond with 25 ng/ml FSH levels, and a six-fold increase in the presence of testosterone (10 µM) (Subramanian and Adiga, 1996a). These studies were validated by using aromatase enzyme inhibitors that block the aromatization of androgens to estrogen by aromatase in chicken liver and oviduct. This treatment reduced RfBP secretion to 85% of control, suggesting that the hormonal stimulation is mediated through in-situ synthesized estrogen. This became more evident when, exogenous estradiol-17-β (10 µM) led to three-fold enhanced secretion of RfBP. Inhibition of FSH by tamoxifen (10 µM) reduced RfBP secretion to 75% of control. Overall, these findings suggest that estrogen regulates RfBP expression in hormonally stimulated Sertoli cells, presumably to function as the carrier of riboflavin to the developing germ cells in rodents (Subramanian and Adiga, 1996a).
In female Bonnett macaques, the systemic RfBP concentration appears to be regulated by plasma estradiol levels during the menstrual cycle as well as early pregnancy (Visweswariah and Adiga, 1988). Interestingly, antibodies raised against chicken RfBP caused pregnancy termination in mice, most likely due to B2-deficiency in the developing fetus. RfBPs, as mentioned earlier have been detected in plasma of male monkeys as well as in rat testicular Leydig and Sertoli cells (Bhat et al., 1995). Similar to their female counterparts, RfBP production in male rats and monkeys is sensitive to estradiol-17-β and FSH levels (Subramanian and Adiga, 1996a; Subramanian and Adiga, 1996b).

Human purified RfBP has been reported to have a molecular weight of 36 kDa and an isoelectric point of 4.1 (Natraj et al., 1988). The presence of RfBP in amniotic fluid of pregnant women has also been revealed (Prasad et al., 1992). Detectable RfBP plasma levels were observed in pregnant women after 4 months of gestation and remained high up to 8 months later. In amniotic fluid, a two-fold higher concentration was reported as compared to RfBP levels in serum at 3-4 months of gestation (Natraj et al., 1988). Despite these reports revealing RfBP in humans, the genetic sequence of such a protein remains to be reported on any public database.

From a translational medicine perspective, a recent study by Rao and colleagues reported significant elevations of serum RfBP in patients with breast cancer (Rao et al., 1999). Earlier, Vaidya and coworkers (Vaidya et al., 1998) reported a decrease in serum B2 levels in breast cancer patients; with baseline B2 serum levels achieved after tamoxifen treatment. Combined, these data suggest that RfBP is up-regulated during
breast cancer with a concomitant decrease in plasma riboflavin levels. Thus, an important role for riboflavin and RfBP in the pathology of breast cancer has been demonstrated, but the physiological role remains to be elucidated. Similar to its potential as a breast cancer biomarker, elevated serum RfBP levels have recently been shown to correlate with hepatocellular carcinoma progression (Rao et al., 2006).

In rats, as stated previously, RfBP synthesis is under the control of estrogen. Knowing the nutritional demand for B₂ during human pregnancy, it can be hypothesized that the biosynthesis of human RfBP could be regulated by estrogen levels. This theory is supported in studies that showed RfBP levels were significantly elevated during the first trimester of pregnancy (Natraj et al., 1988). Furthermore, Clarke and coworkers reported riboflavin levels in cord serum to be 3-4 times higher than in maternal serum, and may reflect a concentrative accumulation of RfBP in response to physiological requirements for B₂ (Clarke, 1977). It is believed that human RfBP may be produced in the liver analogous to avian and rat RfBP. It is regulated by estrogen but the origin of human RfBP in amniotic fluid is not known. It has been proposed that the human placenta synthesizes this RfBP and the liver harbors a RfBP specific mRNA that has the propensity to synthesize this protein, which may also contribute to amniotic fluid RfBP. Natraj and colleagues suggested the presence of human RfBP in amniotic fluid could be due to the direct transfer from maternal circulation through the placenta (Natraj et al., 1988).

Riboflavin concentration in mammalian milk is several fold higher than in maternal circulation during lactation in spite of the presence of circumferential tight
junctions between mammary epithelial cells which constitute a barrier to the free passage of blood constituents (Pitelka and Hamamoto, 1977). Consequently, it was proposed that RfBP synthesis is up regulated by lactating mammary glands to sequester the vitamin for secretion into milk required for neonatal nutrition (Karande et al., 2001). The mammary gland is a known estrogen target tissue, which further strengthens this hypothesis, as estrogen and progesterone are known modulators of RfBP biosynthesis in vivo as well as in vitro (Karande et al., 2001). Interestingly however, a recent report by van Herwaarden and colleagues (van Herwaarden et al., 2007) revealed the multi-drug transporter, breast cancer resistance protein (BCRP/ABCG2, an established [ABC]-transporter), to function in the secretion of B2 into breast milk of lactating mice. These scientists reported a 63-fold reduction in milk secretion of riboflavin in BCRP1−/− lactating females compared to lactating wild-type females. Furthermore, this efflux transporter was shown to be largely instrumental in reducing the tissue distribution and plasma levels for riboflavin, as BCRP1−/− mice revealed substantial reductions (0.8 fold less) and increases (1.8 fold higher) in intestinal and hepatobiliary excretion profiles, respectively. However, pups that were nurtured by standard chow fed BCRP1−/− lactating females only exhibited a modest reduction in plasma riboflavin levels and there were no signs of B2-deficiency. It was only when riboflavin was removed from the diet fed to the BCRP1−/− lactating females that the pups suffered vitamin deficient states, as was evidenced by a lack of growth, severe anemia, and fatty degeneration of the liver. Collectively, this report suggests that BCRP functions in reducing the systemic and tissue levels of riboflavin. However, the
overall effects are modest and other distinct uptake mechanisms are suggested to regulate riboflavin homeostasis to offset the extrusion capacity of this efflux system.

1.4.2.2 Utilizing the riboflavin absorption and trafficking mechanism to enhance macromolecule delivery

Serum albumin is known to adsorb riboflavin and has been demonstrated to co-elute with RfBP during riboflavin affinity purification. Thus, serum albumin is considered a non-specific carrier of riboflavin under normal physiological conditions. In addition, Low and colleagues reported facilitated entry of BSA into human lung, ovary and pulmonary cell systems upon covalently labeling BSA with riboflavin (Wangensteen et al., 1996; Holladay, 1999). These studies were the first to demonstrate the utility of the B₂ absorption pathway in enhancing macromolecule delivery, and suggested such B₂-specific mechanisms to involve endocytosis and transcytosis events. These results were substantiated by experiments revealing 1) a significant reduction in BSA-B₂ uptake in the presence of transcytosis inhibitors and 2) accumulation of these conjugates to organelle populations resembling endosomes. However, these same researchers reported cellular accumulation of BSA-B₂ was inhibited by unlabeled BSA and not by free riboflavin. Overall, in addition to endocytosis, riboflavin absorption is suggested to be regulated by multiple internalization mechanisms.

1.5 Comparison of B₂ internalization with folate uptake and transport mechanisms

Chicken RfBP belongs to the family of folate binding proteins that are glycosylphosphatidylinositol (GPI) anchored proteins, and found on the outer surface of the plasma membrane. Eight of the nine pairs of cysteines involved in disulfide bonds in
RfBP are conserved in milk folate binding protein (FBP), as are all of the tryptophan residues (Zheng et al., 1988). This conservation of cysteine residues indicates the importance of secondary structure and function of cRfBP. Sequence identity between homologous regions of these two vitamin-binding proteins is more than 30% (Zheng et al., 1988). Furthermore, RfBP is evolutionarily conserved in terms of its physiochemical and immunological characteristics and is involved in the embryonic vitamin nutrition from avian to primate species. Folic acid contains a pterin ring structure, which is similar to the B\textsubscript{2} structure. In addition, sequence similarity of the residues in cRfBP and FBP indicate similar folding of the ligand binding domain in the two proteins (Monaco, 1997). Considering these commonalities in binding protein biochemistry and vitamin structural similarities, it was theorized that both vitamins were absorbed via the same cellular entry pathways upon binding to their respective soluble carrier proteins. However, studies performed in our laboratory suggested otherwise, as the internalization of \[^{3}\text{H}]\text{-B}_2\text{ in the presence of folate (1000-fold excess) did not inhibit B}_2\text{ uptake in human placental trophoblasts (Huang and Swaan, 2001). Furthermore, enzymatic studies carried out by our laboratory involving the cleavage of GPI-linked plasma membrane receptors in this same cell model revealed no alteration in \[^{3}\text{H}]\text{-B}_2\text{ uptake (unpublished data). Taken together, these studies suggest the B}_2\text{ and folate uptake mechanisms are distinct, at least in human placental trophoblasts, and thus involve different plasma-membrane associated protein machinery.}
1.6 Summary and thesis objectives

Over the last several decades, our understanding of the molecular regulation and cellular transport mechanism specific to B₂ has significantly increased. In general, riboflavin absorption across divergent human cell systems exhibits the following salient properties: temperature dependence, a general Na⁺-independence, a saturable active transport component that appears to dominate at near basal B₂ concentrations (~ 12 nM), and a passive diffusion component that dominates at concentrations above physiological vitamin concentrations. In addition, the Ca⁺/calmodulin-, protein kinase A and G pathways are suggested to regulate B₂ uptake (Huang and Swaan, 2001). More recently, receptor-mediated endocytosis (RME) has been shown to play a role in riboflavin absorption across the placental barrier (Huang et al., 2003). The role of RfBP in B₂ absorption and transport is still elusive and the existence of a human RfBP analog has not been definitively demonstrated. However, in a recent report by our laboratory that showed nearly complete inhibition in B₂ uptake along human placental trophoblasts (BeWo) treated with antibodies raised against chicken RfBP may suggest the importance of such a carrier protein in regulating the cellular entry of riboflavin (Mason et al., 2006). Furthermore, in light of reports revealing elevated serum levels of RfBP in human breast- and hepatocellular carcinomas (Rao et al., 1999; Karande et al., 2001; Rao et al., 2006), a clinical niche has been suggested for utilizing the riboflavin-specific absorption pathway as an indicator of cancer progression and in targeted chemotherapeutic strategies. However, all studies to date have involved indirect methods to define B₂-specific absorption and trafficking mechanisms in human cell systems. Currently, it is apparent
that there are multiple absorption processes that are regulating this vitamin’s cellular entry. These modes include a passive component and an active component. Regarding its active uptake, it appears that a receptor-mediated pathway plays a role, specifically involving clathrin-mediated endocytosis (CME), in part in non-renal cell systems. However, a transporter component has not been excluded as an alternate route of internalization. Thus, some of the questions remaining to be answered regarding riboflavin uptake and trafficking in human cells include the following: 1) to what extent is this vitamin being internalized by RME or non-RME processes 2) what is the rate at which B₂ is initially absorbed and trafficked to cellular organelles specific to RME and 3) what specific proteins facilitate these RME or non-RME mechanisms? These are important questions that need to be answered to enrich the knowledge base for future studies aimed at utilizing the B₂ internalization pathway in drug targeting approaches. However, this thesis focuses on characterizing the RME mechanisms regulating B₂ internalization and trafficking in human epithelia. Specifically, the major objectives of this thesis include:

1. Define the temporal and spatial distribution of endocytosed B₂ in human epithelial cell models BeWo and Caco-2, both of which are known to exhibit high affinities (nM) for this vitamin,
2. Define the extent by which B₂ absorption and trafficking depends on RME,
3. Elucidate whether B₂–specific RME involves clathrin-independent endocytic pathways, such as the common lipid raft/caveolae pathway.
CHAPTER 2

cAMP-COUPLED RIBOFLAVIN TRAFFICKING IN PLACENTAL TROPHOBLASTS AND ENTEROCYTES: A DYNAMIC AND ORDERED PROCESS

Abstract

Riboflavin (vitamin B₂), an essential micronutrient central to cellular metabolism through formation of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) cofactors, is internalized, at least in part, via a proposed receptor-mediated endocytic (RME) process. The purpose of this study was to delineate the cellular B₂ distribution using human placental trophoblasts and enterocytes, and evaluate the regulatory role of cAMP in this process. Subcellular fractionation and 3D confocal microscopy analyses were carried out to define the B₂ accumulation profile. Biochemical assays evaluating the cAMP dependence of this pathway were also performed. The present study records an intracellular B₂ distribution pattern that shows dynamic
accumulation of the ligand predominantly to the endosomal and lysosomal compartments, and to a lesser extent to the Golgi and mitochondria. In contrast, transferrin (TF) colocalizes rapidly within endosomes with minimal accumulation in the other organelles. Temporal and spatial distribution of B₂ colocalized with unique markers of the endocytic machinery provide added morphological evidence in support of the RME process with ultimate translocation to the mitochondrial domain. Colocalized staining with the Golgi also suggests a possible recycling or exocytic mechanism for trafficking of this ligand. Furthermore, this study demonstrates cAMP regulation of the putative ligand-bound B₂ receptor and its association into endocytic vesicles. Delineating the dynamics of the process governing cellular B₂ homeostasis presents an untapped resource that can be further exploited to improve our current understanding of nutritional biology and fetal growth and development, and perhaps target the endogenous system to develop novel therapeutic approaches.
2.1 Introduction

Dietary intake of free riboflavin (vitamin B₂) or following release from the coenzyme forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) determines the nutritional status of this essential nutrient in humans. Physiological deficiency of B₂ accounts for clinical manifestations of growth retardation, anemia, cardiovascular disease, and neuro-degenerative disorders (Cooperman, 1991; Powers, 2003a). Despite the critical role of flavin analogs in normal cellular metabolic processes, little is known about the mechanism and regulation of the vitamin B₂ absorptive process. Investigative approaches based on biochemical characterization and two-dimensional fluorescence microscopy have identified a putative receptor-mediated component to be responsible, at least in part, for B₂ uptake in human enterocytes and trophoblasts. High ligand-binding affinity (nanomolar range), temperature- and ion-dependence, and a saturable process that can be inhibited by structurally related ligands are the salient features that describe this system (Huang and Swaan, 2000; Huang and Swaan, 2001; Foraker et al., 2003). Analogous to iron recruitment and absorption, the proposed B₂ entry mechanism involves initial recognition by specific binding proteins that chaperone the hydrophilic ligand across membrane barriers within vesicles (Mason et al., 2006). However, the identities of these proteins and other components that facilitate the absorption of this essential nutrient remain elusive. Consequently, elucidating the molecular machinery that contributes to the cellular homeostasis of this vitamin has generated considerable interest in nutritional biology and fetal growth and development. Furthermore, similar to other characterized nutrient absorption pathways, the riboflavin
Internalization mechanism is suggested to be instrumental in serving as a portal of cellular entry that can be exploited to promote targeted drug design and delivery to breast- and liver cancers (Rao et al., 1999; Rao et al., 2006).

Internalization of extracellular nutrients in eukaryotes via receptor-mediated endocytosis employs complex cell machinery in a sequential and regulated manner. The co-existence of abundant intracellular structural and signaling networks offers several possibilities for receptor-associated ligands. Most water-soluble vitamins are fated to metabolic trapping, a term that describes enzymatic processing of the molecule to forms that are spatially restricted within the cell (Aw et al., 1983). However, alternative translocation routes, including late endosomal or lysosomal degradation, membrane recycling, or even transcytosis (in polarized epithelial systems) remain a distinct possibility (Maxfield and McGraw, 2004). Such receptor-ligand interactions also occur under extensive cellular regulation either via an intrinsic mechanism such as kinase or phosphatase activity or via generation of signaling cascades that include second messenger regulation of integrated pathways (Schulte and Fredholm, 2003; Barbieri et al., 2004). Huang and colleagues have demonstrated that the B2 internalization process is affected by modulation of the cyclic nucleotide second messengers, cAMP and cGMP, as well as by protein kinase A, G and calcium-calmodulin pathways (Huang and Swaan, 2001).

The objective of the current study was to evaluate the fate of internalized B2 over time by following its intracellular sorting using the human placental trophoblast and enterocyte cell lines, BeWo and Caco-2, respectively (Pattillo and Gey, 1968; Rindler
and Traber, 1988; Hughson and Hopkins, 1990; Knight et al., 1995), and define the regulatory role of cyclic-AMP in this process. The enhanced nutritional requirements of the fetus (Dancis et al., 1985; Kirshenbaum et al., 1987; Visweswariah and Adiga, 1987a; Dancis et al., 1988) and the presence of high affinity B<sub>2</sub> systems in both BeWo and Caco-2 (Huang and Swaan, 2000; Huang and Swaan, 2001) justify the use of the selected models. Time- and concentration-dependent fractionation analyses of the localized ligand in endosomes, lysosomes, Golgi, and mitochondria were compared with confocal laser scanning microscopy studies examining time-lapsed 3D colocalization between internalized rhodamine-labeled riboflavin (Rd-RF) (Huang et al., 2003; Phelps et al., 2004) and immunolabeled organelles. Trafficking profiles for B<sub>2</sub> were compared with those determined for transferrin (TF), a receptor-mediated endocytic marker, (Dautry-Varsat et al., 1983; Jin and Snider, 1993; van Dam and Stoorvogel, 2002), and for taurocholic acid (TCA) that does not follow a vesicular pathway (Zhang et al., 2002; Zhang et al., 2004). In addition, cyclic AMP assays were included to determine regulation of the downstream events of cellular B<sub>2</sub> translocation upon its binding to the proposed B<sub>2</sub>-receptor. Overall, the study for the first time shows that B<sub>2</sub>, like TF, accumulates mainly within endosomes but differs from TF in the extent of lysosomal distribution. The present study also suggests a possible recycling mechanism that contributes to B<sub>2</sub> homeostasis and regulation via the cAMP signaling pathway in the trophoblast model.
2.2 Methods and materials

2.2.1 Cell culture

BeWo and Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) and routinely maintained in a controlled atmosphere at 37 °C, under 5% CO₂. BeWo (passage numbers 206-221) and Caco-2 (passage numbers 30-48) cells were routinely cultured in F-12K and DMEM medium, respectively (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded in 150 mm culture dishes at a density of $3 \times 10^4$ cells/cm² and were used 5 to 7 days post-seeding for fractionation studies. All studies involved cells in the proliferating phase, as higher nutrient demands have been commonly correlated with rapidly dividing cells (e.g., a characteristic of tumor cells) compared to differentiated cell systems (Ross et al., 1994; Ponka and Lok, 1999).

2.2.2 Labeling of transferrin

Holo-transferrin (Sigma, St. Louis, MO) was labeled with Na-$^{125}$I (~ 5 µCi /µg; Amersham Biosciences, Piscataway, NJ) using the IODOGEN® method (Pierce Biotechnology Inc., Rockford, IL). Iodinated protein was desalted by gel filtration using Micro-Bio-Spin® columns (Bio-Rad Laboratories, Hercules, CA) and $^{125}$I-incorporation was determined by gel electrophoresis and autoradiography. The specific activity of the $^{125}$I-transferrin was ~ 400 cpm/pmol.
2.2.3 Ligand uptake and subcellular fractionation

Cell monolayers were dosed with 10 nM each of $[^3]$H-riboflavin (Sigma, St. Louis, MO) and $^{125}$I-transferrin, or 20 nM $[^3]$H-taurocholic acid (American Radiolabeled Chemicals, Inc., St. Louis, MO), in Hanks’ Balanced Salt Solution (HBSS, pH 7.4) containing 25 mM glucose and 10 mM HEPES at 37 °C for 2 hr. After incubation, cells were washed thoroughly with ice-cold Dulbecco’s phosphate-buffered saline (DPBS) containing Ca$^{2+}$ and Mg$^{2+}$ (pH 3.0) to remove plasma membrane associated ligands. Cells were then pooled in homogenization buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5) and protease inhibitors (Complete Mini®, Roche, USA), and allowed to swell on ice for 15 min prior to lysis through a 25G 5/8 hypodermic needle and monitored by phase-contrast microscopy. The cell lysate was centrifuged at 600 × g for 5 min at 4 °C to yield a nuclear pellet (N, represents nuclear and dense membrane fragments) and postnuclear supernatant (PoN). Total protein content of each fraction was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Endosomes, lysosomes, Golgi, and mitochondria were isolated by loading the PoN fraction on a discontinuous sucrose gradient (0.8 – 2.0 M) and subjected to 205,000 × g for 2 hr at 4 °C using a SW 55Ti rotor (Beckmann Coulter Inc., Fullerton, CA). The gradient was top-fractionated into 350 µl aliquots. Accumulation of radiolabeled ligands in each fraction was measured by a quench-curve corrected dual-label (i.e., $[^3]$H and $^{125}$I) liquid scintillation counting program (Beckmann Coulter Inc., Fullerton, CA) and normalized to total protein content determined by the Bradford assay. Time- and concentration-dependent enrichment of riboflavin and transferrin to these organelles were
assessed by varying incubation times (30 min – 2 hr) and ligand concentrations (5 – 30 nM).

2.2.4 Immunoblotting and data analysis

Cellular organelles in the collected fractions were identified by western blot analyses using monoclonal antibodies (BD Pharmingen, San Diego, CA) directed against organelle-specific protein markers (i.e., Rab5 GTPase and clathrin for early endosomes, LAMP-1 for lysosomes, GM130 for Golgi, and cytochrome c for mitochondria). Fractionated proteins were resolved on 7.5% or 18% Tris-HCl gels, transferred to PVDF membranes (Immun-Blot™, Bio-Rad, Hercules, CA), immunoblotted using peroxidase-conjugated secondary antibodies, and detected using the ECL plus system (Amersham Biosciences, Piscataway, NJ). Sequentially fractionated samples that were positive for a specific protein marker were grouped together to represent that particular organelle population. Subcellular ligand distribution to the individual organelle populations was expressed as a percentage of the total ligand content in the PoN fraction. Due to overlap of marker expression between fractions, the ligand distribution exceeds 100% but serves as a semi-quantitative approach for identifying the ligand trafficking pathway(s) upon internalization.

2.2.5 Immunofluorescence and confocal microscopy

BeWo cells were seeded 3 - 5 days prior (5 × 10^3 cells/cm^2) on collagen-coated BD Falcon™ culture slides (BD Biosciences, Bedford, MA). Following serum starvation, pulse-chase studies with either 500 nM rhodamine-B₂ (Rd-RF) (Phelps et al., 2004) or 30 nM FITC-transferrin (FITC-TF, Sigma, St. Louis, MO) were carried out as previously
described (Huang et al., 2003). Cells were then fixed, permeabilized, and blocked prior to immunolabeling for 2 hr at room temperature to detect organelle markers (clathrin, Rab5 GTPase, LAMP-1, GM130, or cytochrome c). Cells were washed thoroughly and probed with either Alexafluor546®- or Alexafluor405®-labeled sheep anti-mouse IgG (Molecular Probes, Eugene, OR). Immunofluorescence treatments were preserved using GelMount™ (Biomed Corp., Foster City, CA), and visualized using a Nikon Eclipse TE2000 E inverted confocal laser scanning microscope equipped with three fixed lasers, 488-, 405-, and 543 nm (Nikon Instruments Inc., Melville, NY). All 3D confocal images were acquired using the following parameters on Nikon EZ-C1 acquisition software (Gold version 2.3, Image Systems Inc., Columbia, MD): Nikon Plan Apo 60x oil objective (1.4 numerical aperture), 6.00 µs scan dwell, 512 × 512 pixel size, 0.25 µm z-step, 60- or 150 µm detector pinhole, and constant attenuation of laser 488 nm using a neutral density filter (ND8). Images were iteratively deconvolved using a calculated point spread function for each fluorescent channel and corrected using intensity thresholds defined by treatment with either a non-reactive rhodamine derivative or secondary antibodies alone, and by applying a median filter. All images were then analyzed to define 3D colocalized regions using Volocity 3.6 software (Improvision Inc., Lexington, MA). The extent of colocalization between fluorescent ligands and AlexaFluor®-labeled organelles was determined by calculating the percent of total overlapping volume (cubic micrometers) between the two channels compared to the corresponding total ligand volume. All data were averaged using 3 - 4 regions of colocalized distributions and
statistical significance was determined using the Pearson’s Correlation (Manders et al., 1993).

2.2.6 Determination of cyclic AMP accumulation in BeWo cells

BeWo cells were seeded for assays evaluating the cAMP response to the B2 stimulus (2.5 × 10^5 cells/well) and cAMP inhibition assays (1.0 × 10^5 cells/well), one day prior to use in 24-well Biocoat™ plates (BD Biosciences, Bedford, MA). Cells were washed with Earle’s Balanced Salt Solution (EBSS, pH 7.4) and incubated at 37 °C with 10⁻¹² to 10⁻³ M stimulant (i.e., forskolin or riboflavin) for the indicated times in 250 µl of stimulation buffer (20 mM HEPES buffered F-12K, pH 7.4, containing 30 µM Ro-20-1724, a phosphodiesterase inhibitor and 200 µM sodium metabisulfite, an anti-oxidant). Negative coupling assays were carried out by preincubating cells with varying B2 concentrations for 10 minutes prior to stimulating with 1 µM forskolin. The reaction was terminated by discarding the stimulation buffer and adding 200 µl of 3% perchloric acid per well. After incubating on ice for 30 min, 80 µl of 15% KHCO₃ was added to each well, and the plates were further incubated for 10 min. The plates were then centrifuged for 10 min at 1,300 × g. Subsequently, 50 µl of the supernatant from each well was transferred to 250 µl of reaction mixture (150 µl of Tris-EDTA buffer, 50 µl of cAMP-binding protein, and 50 µl of [³H]-cAMP) and incubated at 4 °C overnight. On the following day, 250 µl of charcoal/dextran mix (1%) was added to each sample followed by incubation at 4 °C for 15 min, and then centrifugation for 15 min at 1,300 × g. Radioactivity in the supernatant from each tube was quantified by liquid scintillation
counting. Cyclic AMP concentrations were calculated using a standard curve according to the protocol of the assay kit (Diagnostic Products Corporation, Los Angeles, CA).

2.3 Results

2.3.1 Isolation of organelle-enriched fractions

Cell homogenates following ligand exposure were centrifuged to separate the denser membrane fragments and nuclei from the lower density organelles. Separation of nuclei was confirmed by DAPI staining (Molecular Probes, Eugene, OR). Another polarized epithelial system, the human intestinal Caco-2 cell line, which also exhibits nanomolar affinity for B2 (Huang and Swaan, 2000), was used in parallel. Total intracellular content of riboflavin that accumulated within the fractionated compartments was compared with that of transferrin and taurocholic acid, respectively taken to be positive and negative controls for the receptor-mediated endocytic process. As expected, the BeWo- and Caco-2-associated profiles for TF when expressed as % total dose revealed substantial ligand accumulation (28 – 48 %) independent of cell line. Likewise, the riboflavin load in the fractions was ≥ 8%, but TCA showed poor accumulation, at least in BeWo cells (Figure 2.1). Such distinct distribution profiles for B2, TF, and TCA in cells from different tissue origins provide validation of the isolation procedure.
Figure 2.1. Comparative cell-associated profiles of $[^3]$H-riboflavin (10 nM B$_2$) with the endocytic marker $^{125}$I-transferrin (10 nM TF) and an unrelated bile acid transporter substrate, $[^3]$H-taurocholic acid (20 nM TCA), in placental (BeWo) and intestinal (Caco-2) cells. Ligand content derived from nuclear and postnuclear fractions and expressed as the percent of total dose revealed $\geq$ 8% B$_2$ accumulation (hashed bars), while TF levels (white bars) varied from 48% in BeWo cells to 28% in Caco-2 cells. The distribution profile of TCA (black bars) showed negligible accumulation in trophoblasts versus enterocytes.
Protein content of the nuclear fractions was significantly higher (~ 5 times) than that for postnuclear fractions that had protein yields varying between 2.30 – 4.95 mg/ml. However, the protein load per gradient was kept constant. The identity of the fractionated gradient was determined by western blot analyses for organelle-specific markers. Clathrin heavy chain (180 kDa), a protein component of the endocytic vesicle coat (Nathke et al., 1992), and Rab5 GTPase (25 kDa), a GTP-binding protein associated with early endosomes (Bucci et al., 1992), were detected mainly in fractions 1 through 7 isolated from BeWo cells (Figure 2.2C). Likewise, Caco-2 cells upon subcellular fraction analysis showed a similar distribution of clathrin but Rab5 GTPase was restricted to fractions 1 and 2 (Figure 2.2D). Lysosomal-associated membrane protein (LAMP1; 110 kDa) (Rohrer et al., 1996) indicated the presence of lysosomes in fractions 3 through 10 in BeWo (Figure 2.2C), and 5 through 9 in Caco-2 (Figure 2.2D). Due to overlap in protein markers between fractions, the organelles were not exclusive to any given fraction but instead pooled fractions were considered enriched for a defined subcellular population.

2.3.2 Distribution of internalized B2 and TF

In order to evaluate the intracellular localization profile of B2 after cellular entry, cells were loaded for 2 hrs at 37 °C with 10 nM of each [3H]-B2 and 125I-TF. The endocytic uptake and processing of TF enabling cellular iron transfer has been well-documented (Dautry-Varsat et al., 1983), thereby supporting its use as a marker with which to contrast the trafficking pattern(s) of B2. Following incubation with the ligands, subcellular fractions isolated from trophoblasts and enterocytes by density gradient centrifugation were measured for radiolabeled B2 and TF by liquid scintillation counting.
Subcellular distribution patterns for $[^3]$H-B$_2$ and $^{125}$I-TF measured in the postnuclear organelle fractions from both cell models resulted in localized bands of the radiolabels along the sucrose gradients. B$_2$ was detected mainly in the heavier fractions for both cell lines (fractions 7 – 12 in BeWo, and fractions 8 – 12 in Caco-2), whereas TF appeared to concentrate in the less dense fractions (fractions 2 – 7 in BeWo and 2 – 8 in Caco-2) of the gradient (Figure 2.2A and B). Degradation of $^{125}$I-TF resulting in loss of label during the cell homogenization and fractionation process poses a potential concern. Hence, the integrity of the marker following processing was verified by autoradiography, which revealed intact protein at ~ 83 kDa in all fractions containing transferrin (Figure 2.2C and D). Consequently, the organelle isolation process did not interfere with the compartmentalization and detection of TF as demonstrated for both cell models.
Figure 2.2. Distribution of $[^3]$H-B$_2$ and $^{125}$I-Transferrin (TF) in the postnuclear fractions isolated from trophoblasts and enterocytes. Postnuclear fractions isolated from BeWo (A & C) and Caco-2 (B & D) cells were resolved based on differential organelle densities using a discontinuous sucrose gradient, fractionated, and measured for dual radiolabel accumulation (closed squares - $[^3]$H-B$_2$; open circles - $^{125}$I-TF). Organelle-enriched fractions were identified by Western blot analyses for BeWo and Caco-2 (C & D, respectively) using antibodies directed against endosomal (clathrin, Rab5 GTPase), lysosomal (LAMP-1), and Golgi-associated (GM130) marker proteins. Nuclear fractions (i.e., the denser plasma membrane and nuclear fragments separated from cytosolic or ‘PoN’ contents of cell lysates) were examined for the mitochondrial marker (cytochrome c), as the PoN fractions were negative for the expression of this protein. All protein markers detected in fractions were compared to their corresponding expression in unfractionated whole cell lysate samples (WC). Intact radiolabel on TF following homogenization and gradient fractionation was visualized at ~ 83 kDa by gel electrophoresis and autoradiographic exposure of the isolated fractions from trophoblasts (C) and enterocytes (D).
Labeling of both cell models with 10 nM each of B2 and TF resulted in a
differential rate of ligand buildup. At the end of 2 hrs, B2 exhibited a slower rate of
internalization (i.e., 22.1 – 33.6 pmol/mg prot/hr) relative to TF that accounted for almost
one-third to one-half the administered dose (i.e., 71.5 – 119.7 pmol/mg prot/hr).
Quantitative ligand distribution at 2 hr associated with the organelle-enriched fractions as
determined from marker protein evaluation showed ~ 38 – 50% of postnuclear B2 content
in the endosomes in BeWo cells (Figure 2.3A) whereas endosomal fractions from Caco-2
cells accounted for ~ 7 – 13% of the same ligand (Figure 2.3B). Like B2, TF also showed
increased signal intensities in the endosomal regions (≥ 95%) in BeWo cells at 2 hr
(Figure 2.3C), which is in accordance with previous reports showing TF accumulation
within an acidic nonlysosomal compartment (Ciechanover et al., 1983; Yamashiro and
Maxfield, 1984; Yamashiro et al., 1984). Although lysosomal accumulation of TF in
Caco-2 cells was significantly less than the endosomal levels (Figure 2.3D), B2
distributed in equivalent amounts (~ 20%) between the two organelles populations
(Figure 2.3B).

Ligand-specific differences were detected in their time-dependent endosomal and
lysosomal accrual. In BeWo cells, B2 localization in endosomes (~ 45%) and lysosomes
(~ 10%) within 30 min occurs less rapidly than TF endocytosis (Figure 2.3A and C).
Endosomal and lysosomal accumulation of B2 reached a maximum at the end of 1 hr
(Figure 2.3A) and declined by 30 – 35 % after 2 hr. In contrast, endosomal and lysosomal
content of TF in BeWo cells increased over time with maximal disposition in the
endosomes at 2 hr (Figure 2.3C). Caco-2 cells, on the other hand, demonstrate a more
rapid B₂ internalization process with maximum vesicular localization at 30 min (~ 20%) that declines over 2 hr to ~ 15% (Figure 2.3B). Conversely, TF cargo within the endosomal fractions of Caco-2 cells increases while lysosomal levels decrease over the 2 hr period (Figure 2.3D). Differences in ligand accumulation profiles over time suggest differential requirements for vitamin B₂ or iron that are met via multiple and simultaneous mechanisms in a cell-specific manner. Furthermore, evaluation of the concentration-dependent accumulation of B₂ in BeWo (5 – 25 nM) and in Caco-2 (10 – 30 nM) cells revealed a dose-dependent increase in vesicular buildup of the ligand (Figure 2.4A and B), thus confirming active uptake of B₂ via endocytic machinery.
Figure 2.3. Time-dependent localization of $[^3\text{H}]$-B$_2$ and $^{125}\text{I}$-TF within the separated fractions from BeWo (A & C) and Caco-2 (B & D) cells. Sucrose density gradient fraction analysis by Western blotting of organelle-associated markers (closed squares - clathrin; closed triangles - Rab5 GTPase; open triangles - LAMP1; open circles - GM130; closed circles - cytochrome c) following 30 min, 60 min, and 120 min exposure to $[^3\text{H}]$-B$_2$ (A & B) and $^{125}\text{I}$-TF (C & D) revealed ligand-specific distribution profiles.
Figure 2.4. Concentration-dependent trafficking profiles of $[{}^{3}H]$-B$_2$ after 2 hr incubation at 37 °C in BeWo and Caco-2 cells. Ligand accumulation of 5 – 25 nM B$_2$ (A) and 10 – 30 nM B$_2$ (B) in endosomes (closed squares - clathrin; closed triangles - Rab5 GTPase), lysosomes (open triangles - LAMP1), Golgi (open circles - GM130), and mitochondria (closed circles - cytochrome c) increased in a dose-dependent manner in placental trophoblasts (A) and enterocytes (B).
2.3.3 Colocalization of B₂ with endocytic organelles in BeWo

Cellular distribution of internalized B₂ was further examined using a characterized rhodamine-labeled riboflavin conjugate (Rd-RF) that exhibits affinity characteristics similar to the natural vitamin (Huang et al., 2003). Although the rate of internalized B₂ in the placental trophoblasts is suggested to be slower than that shown for Caco-2, the overall higher ligand accumulation profiles for this vitamin’s enrichment to endosomal populations justify using the trophoblast model over the enterocyte cell system for the remaining studies presented in this report. Since maximal accrual of B₂ within endocytic organelles in BeWo cells occurred after 60 minutes, all fluorescence-based studies were performed using this uptake period. Internalized Rd-RF or FITC-TF were examined for colocalized signals with AlexaFluor405®- or AlexaFluor546®-labeled proteins for endosomes (clathrin or Rab5) and lysosomes (LAMP-1) using confocal laser scanning microscopy. Signal intensities specific to Rd-RF were normalized to cells dosed with equimolar amounts of a non-reactive rhodamine analog, carboxytetramethylrhodamine-4-amine (CTMR4A) (Phelps et al., 2004). In all cases, the signal-to-noise ratio for CTMR4A was significantly less than that for Rd-RF, and, in contrast to Rd-RF, staining patterns were routinely diffuse (data not shown). Like FITC-TF, internalized Rd-RF resulted in distinct punctate, perinuclear staining and exhibited colocalization with both clathrin and Rab5 GTPase (Figure 2.5A and B). In contrast to FITC-TF, Rd-RF also exhibited partial colocalization with the lysosomal marker, LAMP-1 (Figure 2.5C).
Figure 2.5. Endosomal and lysosomal colocalization of Rhodamine-B₂ (Rd-RF) and FITC-labeled transferrin (FITC-TF). Rd-RF and FITC-TF were examined for colocalization with immunostained early endocytic (clathrin (A) and Rab5 GTPase, (B)), and lysosomal (LAMP1, (C)) protein markers after 60 minutes of ligand internalization in BeWo cells. Images represent orthogonal 3D profiles with the inset view defining the XY axis and the outer panels reveal the YZ (right narrow panel) and XZ (upper narrow panel) focal planes. Fluorescence signals for each channel were merged to reveal regions of colocalization (indicated by arrows and yellow regions) and overlayed with the corresponding differential interference contrast (DIC) image to define cell morphology (far right inset view). The nucleus is represented by ‘N’. Scale bars (µm) are defined in the merged inset views.
A quantitative assessment of the extent of colocalization between ligands and endocytic markers revealed maximal signal overlap with Rab5 GTPase for both Rd-RF (64%) and FITC-TF (45%) (Figure 2.6A and B). Compared to the 60 minute fractionation data in figure 2.3A, the total B2 distribution to this endosome population (~70%) is strikingly similar to that seen with Rd-RF. However, the extent of FITC-TF colocalization with Rab5-positive endosomes was nearly 2-fold lower (Figure 2.6B) compared to that defined in the fractionation results in figure 2.3B. This disparity may be attributed to the presence of a heterogenous sub-population of endosomes that are Rab5 negative but TF positive; an incidence that cannot be detected by subcellular fractionation due to the fractional overlap. Rd-RF also revealed ~ 51% colocalization with clathrin, whereas, FITC-TF showed only 8% overlapping signal with this early endosome protein. The Pearson’s Correlation (PC), a common statistical test for shape similarities exhibited between colocalized fluorescence channels, was used to further define linear relationships existing between ligands and organelle markers. Values > 0.0 were defined as having a positive linear relationship between variables (Manders et al., 1993). Interestingly, both ligands revealed positive correlations with all organelle-associated proteins (Figure 2.6). Consistent for both Rd-RF and FITC-TF, the relative colocalization (14% and 6%, respectively) with the lysosome protein, LAMP-1 were markedly less than those determined for each ligand’s association with endosomes. Although both ligands revealed colocalization with LAMP-1, the overall enrichment to lysosomes is suggested to be minimal after the 60 min uptake period. Collectively these results indicate significant ligand recruitment for both B2 and TF to early or sorting endosomes via plasma
membrane-associated clathrin and/or Rab5 positive vesicles (Gorvel et al., 1991; Bucci et al., 1992; McLauchlan et al., 1998). In contrast to TF, the internalized B2 demonstrates higher lysosomal accumulation. Riboflavin that bypasses lysosomal degradation is likely to be sorted to recycling endosomes, similar to apo-transferrin which undergoes exocytosis at the plasma membrane (Jackle et al., 1991).
Figure 2.6. Quantitative evaluation of the 3D colocalized regions of ligands, B2 and TF, with organelle protein markers in BeWo cells. Overlapping volumes (µm$^3$) for either Rd-RF (A) or FITC-TF (B) with organelle channels were expressed as a percentage of the total volume for each ligand. The Pearson’s Correlation (PC) was chosen to define the similarity of 3D shapes between ligands and overlapping organelle marker channels. Data are expressed as the mean ± SEM for 3 - 4 regions of interest.
2.3.4 Golgi and mitochondrial involvement in B2 itinerary

Intracellular enrichment of B2 to the Golgi and mitochondria in comparison with that for TF was also examined in the isolated subcellular fractions. GM130 (130 kDa), a structural marker of the Golgi apparatus (Nakamura et al., 1995) was identified in postnuclear fractions 4 through 6 (Figure 2.2C) while nuclear fractions 11 and 12 tested positive for cytochrome c (15 kDa), an integral component of the mitochondrial respiratory chain (Gonzales and Neupert, 1990) (Figure 2.2C) in placental trophoblasts. A similar organelle enrichment profile was noted with the enterocyte system (Figure 2.2D). Steady-state distribution (2 hr) showed 6 – 8% of B2 (Figure 2.3A) and 30 – 37% of TF (Figure 2.3C) distributed to the Golgi. Interestingly, mitochondrial localization of B2 in BeWo cells peaked at 30 min and declined over 2 hrs (Figure 2.3A), while B2 confined to the mitochondria increased over time in Caco-2 cells (Figure 2.3B). Despite this divergent accumulation profile in the two cell models, maximal mitochondrial B2 content was no greater than 30% of the postnuclear load, whereas TF levels associated with the mitochondria were negligible in comparison (Figure 2.3C and D).

In order to further corroborate the Golgi and mitochondrial fate of B2, 3D colocalization of Rd-RF and FITC-TF by confocal microscopy together with the same organelle markers (i.e., GM130 and cytochrome c) was assessed. Both ligands were shown to colocalize to some degree with these organelle markers (Figure 2.7A and B). As noted with the endocytic proteins, Rd-RF and FITC-TF resulted in punctate and perinuclear colocalization with GM130 and cytochrome c. Image analyses for both ligands revealed a positive Pearson’s Correlation with GM130 and cytochrome c, thus
further suggesting the involvement of these organelles in B₂ and TF trafficking. Rd-RF exhibited a higher degree of colocalization (~34%) with GM130 (Figure 2.6A) that was approximately 2-fold higher than that determined for FITC-TF (Figure 2.6B). FITC-TF also revealed the least amount of colocalization (~8%) with the mitochondrial marker, cytochrome c (Figure 2.6B). In contrast, Rd-RF revealed greater than 3-fold higher overlap (~26%) with cytochrome c (Figure 2.6A). Based on the current understanding of B₂ physiology and the role of its cofactors FMN and FAD in normal metabolic functioning of the cell, distribution to the mitochondria appears to be consistent.
Figure 2.7. Localization of Rd-RF and FITC-TF to the Golgi and mitochondria in BeWo cells. Confocal 3D images of either Rd-RF or FITC-TF after 60 minutes internalization in BeWo cells were analyzed for colocalization (arrows and yellow regions) with Golgi (A) and mitochondria (B). Confocal images are defined by orthogonal profiles as described in figure 2.5, and fluorescence signals for ligands and organelle markers were merged with DIC images (far right inset views). Scale bars represent 10 µm and nuclei (N) are defined in DIC images.
2.3.5 cAMP regulation of B₂ binding and initiation of the endocytic cascade in placental trophoblasts

Cyclic AMP is unequivocally a major short-term regulator that regulates autologous or heterologous receptor-mediated endocytic internalization (Foti et al., 1997). Previous reports have shown that increased levels of cAMP elicited by forskolin, Br-cAMP, and 3-isobutyl-1-methyl-xanthine (IBMX) have resulted in a dramatic reduction in B₂ uptake in BeWo cells (Huang and Swaan, 2001; Foraker et al., 2003). In the present study we sought to evaluate if this cAMP-sensitive endocytic trafficking of B₂ occurred as a consequence to regulation of B₂ binding with its specific receptors. BeWo cells stimulated with increasing concentrations of forskolin resulted in a robust cAMP response with an EC₅₀ of 1.5µM [0.99-1.9] and a Bₘₐₓ value of 125 ± 2.2 pmol cAMP/250,000 cells, while treatment with riboflavin up to 10⁻⁴ M failed to stimulate any cAMP accumulation (Figure 2.8A). Interestingly, pretreatment of BeWo cells with increasing concentrations of B₂ for merely 10 min prior to stimulation with 1µM forskolin in the continued presence of B₂ resulted in a diminished cAMP response (Figure 2.8B). The maximum inhibition was ~ 67% of control with a reported IC₅₀ value of ~ 0.4 µM. This indicates that B₂ bound to the putative riboflavin receptor is negatively coupled to cAMP and suggests that stimulated cAMP production, consequently results in decreased B₂ uptake via clathrin-coated pits.
Figure 2.8. Cyclic-AMP regulation of the B₂ internalization process. Determination of cAMP accumulation in BeWo cells after stimulation with riboflavin or forskolin (A). BeWo cells cultured as described in the experimental section were stimulated via incubation with the indicated concentration of either forskolin (closed squares) for 10 minutes, or riboflavin (open circles) for 40 minutes. Data are a representative curve from three independent experiments showing similar results. The inhibitory effects of riboflavin on forskolin-stimulated cAMP accumulation were analyzed (B). BeWo cells were washed and then incubated with the indicated concentrations of riboflavin dissolved in HF12K (20 mM HEPES buffered F12K, pH 7.4, 37 °C). After 10 minutes HF12K was removed and cells were stimulated with 1µM forskolin in the continued presence of riboflavin. Cyclic-AMP accumulation was then determined as described in the experimental section. The dashed line indicates 1µM forskolin stimulated accumulation in the absence of riboflavin. Results are expressed as mean ± S.D. (n = 5).
2.4 Discussion

Our laboratory has recently demonstrated that intestinal and placental entry of vitamin B₂ into the cell occurs via a putative receptor-mediated endocytic process (Huang and Swaan, 2000; Huang and Swaan, 2001; Huang et al., 2003). This B₂-specific endocytic pathway is still in its nascent stage of identification and has warranted investigative pursuits to clearly define its translocation pathway within the cell. In the present study we delineate the trafficking route of B₂ upon internalization that consequently, contributes to maintaining the balanced vitamin status in a physiological environment. In addition, we also report second messenger regulation of the ligand absorption into placental trophoblasts that suggests interaction of other extrinsic pathways in response to the ligand stimulus.

The approach employed followed a conventional fractionation analysis of intracellular compartments that are integral to the ‘classical’ clathrin-dependent endocytic machinery. The BeWo cell line exhibited distinctive accumulations for B₂ and TF within the endocytic system but negligible levels for a non-endocytic marker TCA, which is routinely transported by placental bile acid transporters (Dumaswala et al., 1993). Sucrose density gradient-resolved endosomal and lysosomal fractions defined by widely used organelle markers (clathrin, Rab5 GTPase, and LAMP1) showed extensive labeling for B₂ and TF within the endosomes. Selective enrichment of the endosomes over lysosomes for both ligands was in accordance with previously determined trafficking profiles for TF (Ciechanover et al., 1983; Yamashiro and Maxfield, 1984; Yamashiro et al., 1984) and confirmed the efficiency of the fractionation method. The method also
provided insight into the possible fate of B2 following internalization. Biochemical association with the mitochondrial compartment is expected since FMN and FAD play a pivotal role in the electron transport chain (Barile et al., 2000) generating the metabolic energy required for other cellular functions.

Both placental trophoblasts and enterocytes exhibited comparatively higher uptake levels for TF than B2. This may be attributed to the differential nutritional needs of these cells for iron and vitamin B2. van der Ende and colleagues (van der Ende et al., 1987) have demonstrated that the augmented process of iron transfer and metabolism in the developing fetus is efficiently mediated by the transplacental TF system. Likewise, gestational needs of B2 are also increased (Powers, 2003a) and concomitant fortification of B2 supply would be expected. Further, a time-associated increase in ligand build-up was observed for B2 and TF within the endocytic compartments. The endocytic process entails rapid internalization of recognized ligands; consequently evaluation of time points shorter than 30 min are currently underway. Nevertheless, this method provides an informative overview of the trafficking itinerary of B2 via the endocytic route, which appears to be conserved across the two divergent cell systems applied in this study. The kinetic distribution of 5 – 30 nM B2 expectedly showed increased accumulation within the organelles examined with the exception of mitochondrial compartments. Clearly, the changing distribution patterns of B2 within the mitochondria suggest a tightly-regulated feedback mechanism that controls the trafficking route of the molecule, which in turn maintains a homeostatic check on the cellular riboflavin content. It is very likely that the increased B2 accumulation over time provides the impetus to rapidly convert B2 to the
flavin intermediates that cycle through the metabolic pathway. This dynamic change in compartmentalized B2 presents a challenging scenario of putative vitamin sensors, receptors, and other regulators that must interact to maintain a balanced nutritional environment.

A limitation of any fractionation method is the potential overlap between the organelles identified. Consequently, to further corroborate the intracellular distribution profiles of B2, a minimally invasive labeling approach examining the colocalization of fluorescently labeled ligands with immunostained organelle markers was utilized. Rhodamine-conjugated riboflavin, a derivative of the natural ligand previously characterized in our laboratory (Phelps et al., 2004), that retains affinity for the B2 transport system was compared with the staining patterns of FITC-TF. Rd-RF convincingly localized within the endosomes, lysosomes, Golgi, and mitochondria, whereas FITC-TF was confined predominantly to the endosomes. It is not clear at this time what the significance of localization to the Golgi may signify, but this may reflect a possible recycling mechanism that recovers B2 and packages it either for storage or exocytosis. Evidence for Golgi processing of the TF-receptor is seen in myeloma cells and supports the recycling theory of this receptor that continuously scavenges iron-bound TF (Woods et al., 1986). In fact, prior studies reveal two types of recycling pathways, i.e., a short pathway involving early endosomal fusion with recycling endosomes that target ligands back to the plasma membrane, and a long pathway that translocates ligands to the Golgi at which clathrin initiates vesicular budding from the trans-most cisternae and eventual shuttling to the plasma membrane (Ghosh and Maxfield, 1995; Sakai et al.,
A recycling component specific to the B₂ trafficking itinerary has yet to be defined; however, it would be interesting to delineate whether one or both of these recycling events is specific to this vitamin. It should be noted that the possibility of equilibrative changes associated with time-resolved imaging cannot be ruled out at this juncture and require real-time imaging approaches. However, the intent of this study was to illustrate the involvement of the endocytic machinery in the cellular trafficking itinerary of B₂.

The presence of a receptor-mediated endocytic component in the placental and intestinal internalization of B₂ then raises several questions that need to be addressed regarding its effectors and regulators. Literature reports have established that the endocytic process in eukaryotes occurs through a highly ordered process and is driven by signaling events that activate or inactivate depending upon the stimulus (Foti et al., 1997). Cyclic AMP is clearly a pivotal candidate integral to a large number of signaling cascades and was evaluated as a key regulator of the B₂ endocytic process in the trophoblast model. B₂ alone did not generate a cAMP response but it was responsible in down-regulating the cAMP production upon stimulation with forskolin. This indicates that the ligand-sequestered B₂-receptor lowers the intracellular levels of cAMP prior to or shortly after its association into clathrin-coated pits, and eventually triggers the subsequent endocytic events of sorting and translocation. Conversely, we have already shown that increased stores of intracellular cAMP significantly inhibit the internalization of B₂ (Huang and Swaan, 2001), thereby indicating an inverse relationship between intracellular cAMP and B₂ receptor-mediated endocytosis.
In conclusion, fractionation data in both divergent cell systems taken together with the morphological assessment of cellular B$_2$ distribution provides definitive evidence for endocytic trafficking of B$_2$, at least in part via a clathrin-dependent pathway, and eventual translocation to the mitochondria where the role of its metabolites are well characterized. In addition, this cellular absorption mechanism is shown to be sensitive to cAMP levels, further suggesting the existence of a highly regulated B$_2$-specific-receptor internalization process. Understanding the characteristics of the conserved B$_2$ translocation pathway provides a novel platform that can be potentially exploited in the future, analogous to the folate and transferrin receptors, in biomedical imaging (Berry et al., 2004), and tumor-targeted delivery of genes or therapeutic conjugates (Li et al., 2003; Hattori and Maitani, 2004; Yoo and Park, 2004a; Yoo and Park, 2004b).
CHAPTER 3

DYNAMIN 2 REGULATES THE ENDOCYTOSIS OF RIBOFLAVIN IN HUMAN EPITHELIA

Abstract

Riboflavin (vitamin B\textsubscript{2}) is known to be indispensable in a multitude of metabolic pathways through its conversion to coenzyme forms flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Despite its physiological importance, little is known about specific mechanisms or proteins involved in regulating its cellular entry in humans. Recent evidence suggests riboflavin internalization and trafficking in human epithelia occurs through a clathrin-dependent receptor-mediated endocytic (CME) process. The purpose of this study was to define the role of the conserved endocytic protein, dynamin 2 GTPase (DNM2), in riboflavin absorption into placental trophoblasts (BeWo) using RNA interference (RNAi) and transient transfections with wild-type or GTPase-null dynamin 2 constructs. The extent of plasma membrane bound and internalized
B₂ were elucidated under knocked down, functional, and non-functional dynamin 2 states. Analogous to the endocytic control ligand, transferrin, riboflavin was shown to exhibit ~50% dependence on the functional expression of dynamin 2 for its active cellular entry. Furthermore, a reduction in functional dynamin 2 levels led to ~2 fold higher plasma membrane bound B₂ over its internalization. These data are the first to identify the functional requirement of dynamin 2 GTPase in the B₂-specific RME process in humans. Delineating the mechanism and proteins regulating B₂-RME provides future studies with a novel pathway that may be potentially exploited in targeted drug design, delivery and biomedical imaging strategies.
3.1 Introduction

Riboflavin (vitamin B₂) is an essential nutrient required in many metabolic pathways critical in normal cellular growth and development. States of physiological B₂-deficiency have been correlated with various clinical manifestations including cardiovascular disease, stunted growth, anemia, neurodegenerative disorders and various forms of cancer (Cooperman, 1991; Powers, 2003b; Powers, 2005). Despite its clinical importance, the specific cellular mechanism(s) regulating B₂ absorption are poorly defined. Recent reports by our laboratory suggest one portal of cellular entry involves clathrin-mediated endocytosis (Huang and Swaan, 2000; Huang and Swaan, 2001; Huang et al., 2003; D'Souza et al., 2006b). These studies demonstrated a microtubule dependence and early clathrin and Rab5 positive endosomal enrichment of B₂ in human placental trophoblasts (BeWo). As both clathrin and Rab5 are conserved endocytic markers of the clathrin-dependent endocytic (CME) pathway, this data strongly suggests riboflavin absorption involves CME. Furthermore, analogous to the iron carrier protein, transferrin, a riboflavin carrier protein has been proposed to sequester free extracellular B₂ and facilitate internalization and localization to endosomal organelles (Ramana Murty, 1982; Viswesvariah and Adiga, 1987a; Prasad et al., 1992; Subramanian and Adiga, 1999; Mason et al., 2006). However, a limitation to this B₂-specific RME model is a lack of direct evidence identifying the functional dependence on critical proteins regulating this absorption mechanism in humans.

Dynamin 2 (DNM2) is a ubiquitously expressed GTPase that has been thoroughly established to regulate the invagination and constriction of endocytic vesicles at the
plasma membrane of mammalian endothelial and epithelial cells (Sever et al., 2000; Hill et al., 2001; Sever, 2002; Conner and Schmid, 2003). It is required for multiple endocytic mechanisms including those associated with classical pathways dependent on clathrin or caveolae, and consequently it has been described as the “…master regulator of membrane trafficking events at the cell surface.” (Conner and Schmid, 2003). Prior studies performed in our laboratory revealed distinct subcellular distribution of radiolabeled and fluorescently labeled riboflavin to endocytic populations positive for both clathrin and the early endosome marker, Rab5 GTPase, in placental trophoblasts (D'Souza et al., 2006b). Therefore, we hypothesized that riboflavin internalization requires the functional expression of dynamin 2 GTPase. In order to test this hypothesis, we used RNA interference (RNAi) strategies to knock down DNM2 protein expression in the BeWo cell system, which has been established to exhibit nM affinity for riboflavin (Huang and Swaan, 2001).

Like DNM2, dynamin-like protein 1 GTPase (DLP1) is a member of the dynamin superfamily (Praefcke and McMahon, 2004). DLP1 shares roughly 36% sequence homology to DNM2. Unlike DNM2, DLP1 is not involved in endocytic vesicle scission events. However, DLP1 has been largely characterized to function in the morphological maintenance of peroxisomes and mitochondria (McNiven et al., 2000; Yoon et al., 2003; Pitts et al., 2004). In addition, DLP1 has been implicated as a regulator of exocytic trafficking of vesicles from the Golgi to the plasma membrane (Yoon et al., 1998). Duplex short interfering RNA (siRNA) targeting DLP1 was used to silence DLP1 expression in BeWo cells, and served as a control for siRNA silencing specificity in these
studies. Based on the current understanding of DLP1, we hypothesized this protein is not involved in the endocytosis of riboflavin.

The purpose of this study was to define the extent by which riboflavin absorption depends on endocytosis as a function of the expression level of the major vesicle invagination/scission protein, dynamin 2 GTPase. RNAi combined with wild-type and GTPase-null dynamin 2 transfections in the BeWo cell model were carried out to illustrate the importance of functional dynamin 2 expression on the cellular entry of this important nutrient. Results from this study are the first to directly reveal the dependence of B_2 absorption on dynamin 2 expression in human placental trophoblasts, and demonstrates that approximately 50% of actively absorbed B_2 involves an RME process.

3.2 Methods and materials

3.2.1 Cell Culture

Human placental trophoblasts (BeWo) were routinely maintained in a controlled atmosphere at 37 ºC with 5% CO_2 in F-12K culture media (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin. All transfection treatments were tested for cytotoxic effects using the lactate dehydrogenase-based CytoTox-ONE™ assay according to manufacturer’s instructions (Promega Corp., Madison, WI). All chemicals used in these studies were purchased from Sigma (St. Louis, MO) unless stated otherwise.

3.2.2 Duplex siRNA transient transfection

Cells grown to ~ 50-60% confluency were seeded (4.2 x 10^4 cells/cm^2) onto 24-well plates using antibiotic-free F-12K media. Twenty four hours later, cells were briefly
washed and pre-incubated in serum-free and antibiotic-free Opti-MEM I media (Invitrogen Life Technologies, Carlsbad, CA) for ~ 2 hours at 37 °C and under 5% CO₂. Following this incubation, cells were transfected in Opti-MEM I media with 40 nM duplex siRNA complexed with Lipofectamine™ 2000 (0.25% v/v) according to manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad, CA). Cells were incubated with siRNA-lipid complexes at 37 °C and 5% CO₂ for 6 hours, at which time the transfection media was replaced with normal F-12K media lacking antibiotics. Cells were used in experiments 48-72 hours post-transfection. All RNA interference studies involved the use of experimentally validated duplex siRNA targeting the human dynamin 2 gene (DNM2 pool siGenome Smartpool, gene accession no. NM_004945, Dharmacon RNA Technologies, Chicago, IL) and human dynamin-like protein 1 (DLP1, gene accession no. NM_012063, Qiagen®, Cambridge, MA). Targeting siRNA effects were normalized to cells transfected with equivalent amounts of non-targeting duplex siRNA (Dharmacon RNA Technologies, Chicago, IL) and compared to mock (Lipofectamine™ 2000 alone) and untreated cell conditions.

3.2.3 Western blotting and chemiluminescence-based densitometry

Whole cells transfected 63-68 hours prior with siRNA were harvested and lysed on ice for 20 min in RIPA buffer (10 mM Tris-HCL, pH 7.5, 140 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate and 0.1% (w/v) SDS) supplemented with a Complete Mini® protease cocktail tablet according to manufacturer’s instructions (Roche, USA). Ten to 15 µg total protein was resolved on 12.5% Tris-HCl Criterion gels (BIO-RAD, Hercules, CA), transferred to PVDF membranes, and was immunoblotted using
monoclonal antibodies specific to dynamin 2 (Calbiochem®, EMD Biosciences Inc., San Diego, CA) or dynamin-like protein 1 (BD Pharmingen, San Diego, CA). Primary antibodies were subsequently labeled with horse radish peroxidase-conjugated IgG and detected using the ECL plus system (Amersham Biosciences, Piscataway, NJ). Protein levels were quantitated using chemiluminescence-based densitometry on a chemi-doc universal hood II system (Bio-Rad Laboratories, Hercules, CA) and normalized to the corresponding housekeeping protein expression for either GAPDH (Ambion Inc., Austin, TX) or β-actin.

3.2.4 GTPase-null (K44A) and wild type dynamin 2 plasmid transient transfections and cotransfections with DNM2 pool siRNA

Characterized wild type (DNM2<sup>WT</sup>) and dominant-negative (DNM2<sup>K44A</sup>) dynamin 2 expression constructs (both in the mammalian expression vector, pCR3.1, Invitrogen Life Technologies, Carlsbad, CA) were kindly provided by Dr. Mark A McNiven (Mayo Clinic and Foundation, Rochester, Minnesota) (Cao et al., 1998). Prior to transfections, both plasmids were transformed into DH5-α cells (Invitrogen Life Technologies, Carlsbad, CA), subcultured in Luria broth with ampicillin selection, and purified using the Plasmid Maxi Kit (Qiagen®, Cambridge, MA). Expression vector identities were confirmed through restriction enzyme digests and sequencing using the following established dynamin 2 primers: forward primer [5’-GAAGAGGGCCATACC-3’] and reverse primer [5’-AGTTGCGGATGGTCTC-3’] (Cao et al., 1998). Cell seeding conditions were maintained as in RNAi studies. Twenty four hours post-seeding, cells were briefly washed and pre-incubated in serum-free and antibiotic-free Opti-MEM I for
2 hours at 37 °C and under 5% CO₂. Cells were transfected using Opti-MEM I with 400 ng plasmid DNA alone or with 40 nM DNM2 pool siRNA and complexed with Lipofectamine™ 2000 (0.25% v/v). Cells were exposed to these transfection complexes for 6 hours at 37 °C and under 5% CO₂. Subsequently, the transfection media was replaced with complete F-12K media devoid of antibiotics. Cells were used in experiments 48-72 hours post-transfection.

3.2.5 Radio-labeled ligand endocytosis assays

Cells were dosed with either 5 nM [³H]-riboflavin ([³H]-B₂) or 10 nM ¹²⁵I-transferrin (¹²⁵I-TF, iodinated using the IODOGEN® method (Pierce Biotechnology Inc., Rockford, IL) according to established procedures (D'Souza et al., 2006b)) in Hanks’ Balanced Salt Solution (pH 7.4) containing 25 mM D-glucose and 10 mM HEPES at 37 °C for 4 min. Immediately, cells were placed on ice and free ligands were removed by washing 3x with ice-cold DPBS (pH 7.4). Plasma membrane bound ligands were then removed by washing cells 2x on ice (5 min/wash) with ice-cold DPBS (pH 3.0). Cells were alkaline-lysed (1 N NaOH) at 4 °C for at least 2 hours prior to internalized ligand quantitation. The extent of plasma membrane bound and internalized [³H]-B₂ and ¹²⁵I-TF was determined using liquid scintillation- or gamma counting, respectively. Both, plasma membrane bound and internalized radio-labeled ligands were normalized to total protein content using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). All [³H]-B₂ uptake data generated at 37 °C was corrected for passively absorbed B₂ by performing parallel uptake assays exclusively at 4 °C. Actively (uptake at 37 °C) internalized B₂ was defined by subtracting internalized [³H]-B₂ at 4 °C.
Acid wash samples collected after uptake assays represented plasma membrane bound ligands and were compared to internalized samples using the following equations:

\[
\%\text{bound} = \frac{b_e}{b_e + i_e} \cdot \frac{b_{nt} + i_{nt}}{b_{nt}} \times 100 \quad \text{(Eq. 1)}
\]

\[
\%\text{absorbed} = \frac{i_e}{b_e + i_e} \cdot \frac{b_{nt} + i_{nt}}{i_{nt}} \times 100 \quad \text{(Eq. 2)}
\]

The extent of bound ligand was expressed as a percent of the sum of bound \((b_e)\) and absorbed \((i_e)\) ligand under experimental conditions, and was normalized to the percent of bound ligand under non-targeting conditions \((b_{nt})\) (equation 1). Likewise, the extent of absorbed ligand \((i_e)\) was defined as a percent of bound plus internalized ligand under experimental conditions and then was normalized to non-targeting effects \((i_{nt})\) (equation 2).

### 3.2.6 Statistics

One-way ANOVA with Dunnett’s or Neuman-Keuls multiple comparison tests were used to define statistical significance between the effects of targeting siRNA or wild type and dominant negative dynamin 2 construct treatments and control (i.e., non-targeting siRNA, empty vector, mock, and untreated treatments) conditions on ligand trafficking and targeted protein knockdown.
3.3 Results

3.3.1 Duplex siRNA target specificity at the protein level and RNAi method validation

BeWo cells were transiently transfected with 20-40 nM targeting duplex siRNA for dynamin 2 GTPase (DNM2 pool) or dynamin-like protein 1 (DLP1). Cells were harvested 63-68 hours after initiating siRNA transfections and analyzed for target protein knockdown of dynamin 2 (DNM2) and the dynamin homologue, dynamin-like protein 1 using Western blotting and chemiluminescence-based densitometry. Both DNM2 and DLP1 protein levels were normalized to the housekeeping expression of GAPDH or β-actin, respectively. Upon normalization, the extent of dynamin expression was defined as a percentage of detected dynamin levels in cells treated exclusively with non-targeting siRNA (NT). Parallel cell populations that were treated exclusively with the lipid transfection reagent (Mock) or untreated cells served as additional negative controls.

Twenty and 40 nM DNM2 pool siRNA treatments substantially reduced dynamin 2 (100 kDa) (figure 3.1A) expression levels to a similar extent. Furthermore, quantitative densitometry tests revealed 40 nM DNM2 pool siRNA treatments led to ~78% (21.95 ± 7.03% NT) and ~82% lower dynamin 2 GTPase levels compared to non-targeting siRNA treated and untreated cells, respectively (figure 3.1C). Likewise, 20 and 40 nM DLP1 siRNA specifically knocked down DLP1 protein (83 kDa) expression in a dose-dependent manner (Figure 3.1B). Forty nM DLP1 siRNA conditions resulted in ~62% (37.65 ± 11.13% NT) and ~63% lower DLP1 protein levels compared to non-targeting siRNA and untreated conditions, respectively (figure 3.1D). Neither of the dynamin
siRNA treatments revealed significant off-target effects at the protein level. Furthermore, dynamin protein levels were shown to be similar between non-targeting siRNA treatments and untreated cell conditions. This data suggests the negative control siRNA transfection effects are not significantly altering target protein levels, and serves as a valid negative control for targeting siRNA data normalization.
Figure 3.1. Specific protein knockdown as a function of siRNA treatment. Cells transfected with duplex siRNA targeting mRNA for dynamin 2 (DNM2 pool), or dynamin-like protein 1 (DLP1) were examined for target protein knockdown using western blotting and quantitative chemiluminescence-based densitometry, and normalized to housekeeping proteins, GAPDH or β-actin. Twenty and 40 nM targeting siRNA (A: DNM2p, and B: DLP1) treatments were compared to 40 nM non-targeting (N) siRNA, mock, and untreated (U) cells. Cells treated with 40 nM targeting or non-targeting siRNA were assessed for the extent of DNM2 (C) or DLP1 (D) knockdown, and expressed as a percentage of non-targeting siRNA effects. The data represents the standard deviations of 2 to 4 separate experiments. Statistical significance between targeting and non-targeting effects on dynamin protein expression were defined by one-way ANOVA with Dunnett’s multiple comparison test (**p<0.01).
In order to evaluate whether cell physiology was compromised due to transfection conditions, all treatments were assessed for potential cytotoxicity as a function of lactate dehydrogenase (LDH) release. Cells treated with the various RNAi conditions, along with plasmid transfections, mock, and untreated cell populations, were tested for LDH release 48 hours post-transfection. As shown in Table 3.1, no significant cell death was revealed for any of the transfection treatments, and cell viability for these conditions was at or near those measured for untreated BeWo cells. Taken together, these data indicate that siRNA treatments at the 40 nM dose specifically knock down target dynamin proteins without compromising cell viability, and justify the RNA interference methodology (Figure 3.1, Table 3.1).
Table 3.1. siRNA or plasmid and transfection reagent effects on cell viability as a function of lactate dehydrogenase (LDH) release. All transfection conditions were examined for cytotoxic effects on BeWo cells. Upon 48 hours post-transfection cell viability was assessed using the LDH release assay according to manufacturer’s instructions (Promega Corporation, Madison, WI). These data represent the mean percent of untreated cell viability ± standard deviations of 2 to 4 separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% untreated ± SD</th>
<th>Treatment</th>
<th>% untreated ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLP1 siRNA</td>
<td>104.71 ± 4.22</td>
<td>Empty vector</td>
<td>100.90 ± 6.00</td>
</tr>
<tr>
<td>DNM2 pool siRNA</td>
<td>99.36 ± 10.36</td>
<td>DNM2K417 vector</td>
<td>104.71 ± 0.33</td>
</tr>
<tr>
<td>Non-Targeting siRNA</td>
<td>100.69 ± 2.19</td>
<td>DNM2K444A vector</td>
<td>100.62 ± 1.97</td>
</tr>
<tr>
<td>Mock</td>
<td>100.23 ± 1.55</td>
<td>DNM2 pool siRNA + DNM2K444A</td>
<td>100.40 ± 3.71</td>
</tr>
</tbody>
</table>
3.3.2 Dynamin siRNA effects combined with temperature dependence on endocytosis of \(^{3}\text{H}\)-B\(_{2}\) in BeWo

It is thoroughly established that riboflavin gains entry into human epithelial cells via multiple mechanisms, including a passive diffusion component that appears to dominate at oversupplemented riboflavin levels and an active component that has been reported to coincide with nM B\(_{2}\) concentrations (Huang and Swaan, 2000; Huang and Swaan, 2001). One of the salient features defining an active absorption mechanism is temperature dependence. Saturable absorption kinetics have been consistently shown to correlate with physiological temperatures (~ 37 °C), while low temperatures (~ 4 °C) generate linear absorption profiles reflecting passive diffusion (Said and Ma, 1994). In fact, B\(_{2}\) transport has been reported to be dependent on temperature in divergent cell models (Said and Ma, 1994; Huang and Swaan, 2001). However, temperature dependence alone does not discriminate between a carrier/transporter or receptor-mediated endocytic process. Therefore, in order to differentiate between such active uptake mechanisms, the standardized RNAi methods involving siRNA-induced silencing of the conserved endocytic protein, dynamin 2 GTPase, in combination with the effects of temperature change on B\(_{2}\) absorption were investigated. To date, the human placental trophoblast cell model (BeWo) has been shown to express the highest affinity for riboflavin (~ 2 nM) (Huang and Swaan, 2001), and such nM affinities further suggest the involvement of RME in this vitamin’s cellular uptake. Combined these data validate the utility of the BeWo cell model to characterize the B\(_{2}\)-specific RME pathway(s). BeWo cells transfected with 40 nM targeting or non-targeting siRNA 63-72 hours prior were
used in endocytosis assays. Transiently transfected cells were dosed with 5 nM $[^3]$H-B$_2$ for 4 min at 4 °C or at 37 °C. Immediately following this incubation, cells were placed on ice and free ligand was removed with several ice-cold neutral (pH 7.4) saline washes. Plasma-membrane bound B$_2$ was then collected using acidic saline washes (pH 3.0), and whole cells were alkaline lysed and examined for internalized $[^3]$H-B$_2$ using liquid scintillation counting. Actively internalized B$_2$ was defined by the amount of absorbed riboflavin at 37 °C minus absorbed B$_2$ at 4 °C. Passively absorbed riboflavin was reflected by detected vitamin uptake at 4 °C, exclusively. In contrast to all other transfection conditions, DNM2 pool siRNA-induced silencing of dynamin 2 GTPase resulted in a significant reduction by 50% (i.e., 0.18 ± 0.06 SEM and 0.36 ± 0.05 SEM pmol/mg prot/4 min for DNM2 pool siRNA treated and untreated cells, respectively) in absorbed riboflavin compared to internalized B$_2$ in untreated cell populations (Figure 3.2A). As expected, passively absorbed B$_2$ (i.e., uptake at 4 °C) was unaffected by all transfection conditions (Figure 3.2B). Collectively, these results further substantiate the involvement of classical RME machinery, i.e., dynamin 2 GTPase, in regulating actively absorbed riboflavin in placental trophoblasts.
Figure 3.2. Effect of 40 nM siRNA on riboflavin internalization at 4 °C and 37 °C in BeWo. BeWo cells transfected 63-72 hours prior with 40 nM siRNA duplexes were dosed with 5 nM [³H]-B₂ for 4 min at 37 °C or 4 °C. Actively internalized B₂ (uptake at 37 °C, A) was determined by subtracting passively absorbed riboflavin (uptake at 4 °C, B). This data represents the standard deviations of 3 to 5 separate experiments. Statistical significance between targeting and non-targeting treatments was defined using one-way ANOVA with Newman-Keuls multiple comparison test (*p < 0.05).
3.3.3 Determination of the extent of reduced absorption for B$_2$ compared to the control ligand, transferrin, under silenced dynamin 2 conditions

Iodinated transferrin ($^{125}$I-TF or TF) was chosen to serve as a positive control ligand in all endocytosis assays. Transferrin is an iron carrier protein that has been thoroughly characterized to be internalized via the classical clathrin dependent endocytic pathway, which is known to be regulated by dynamin 2 expression in A431 and HeLa cells (Schmid and Smythe, 1991; Lamaze et al., 1993; Baba et al., 1995). Thus, transferrin uptake is expected to be inhibited to a certain extent under DNM2 pool siRNA treatments, exclusively. BeWo cells were transiently transfected with 40 nM targeting or non-targeting siRNA 63-72 hours prior to using them in uptake assays. Cells were then dosed with either 5 nM [$^3$H]-B$_2$ or 10 nM $^{125}$I-TF for 4 min at 37 °C. Passively absorbed B$_2$ was defined by performing a parallel uptake assay with cells dosed with 5 nM [$^3$H]-B$_2$ for 4 min at 4 °C. As before, data defining passively absorbed riboflavin was subtracted from the results for internalized B$_2$ detected from uptake assays performed at 37 °C to define the active component. The effects of all treatments, including mock and untreated conditions, on ligand absorption were defined as the percentage of non-targeting siRNA effects. Interestingly, both actively internalized B$_2$ and TF absorption were significantly reduced to a similar degree. Under silenced dynamin 2 conditions, riboflavin internalization reduced by ~ 40% (59.45 ± 24.70% of NT, Figure 3.3A) and transferrin absorption reduced by ~ 30% (69.64 ± 4.27% of NT, Figure 3.3B). The attenuation in transferrin uptake seen exclusively with DNM2 pool siRNA treated cells is in agreement with literature reports revealing this ligand’s dependence on clathrin mediated
endocytosis and dynamin 2 expression (Cao et al., 2003), and further validates the
specificity of the RNAi methodology. A similar effect noted with riboflavin absorption
under these same conditions further corroborates prior evidence suggesting this vitamin is
internalized via clathrin dependent endocytosis (Huang and Swaan, 2000; Huang and
Swaan, 2001; Huang et al., 2003; Phelps et al., 2004; D'Souza et al., 2006a; D'Souza et
al., 2006b; Mason et al., 2006). Furthermore, the reduced absorption of riboflavin (40%)
correlated with ~ 80% reduced protein expression for DNM2 (Figure 3.1C). Thus, we can
approximate that 50% of the active component regulating riboflavin absorption in human
placental trophoblasts requires dynamin 2-dependent RME events.
Figure 3.3. Effects of siRNA treatments on the percent of internalized ligand normalized to non-targeting conditions. In this analysis, the overall effect of all treatments is expressed as a percent of non-targeting siRNA effects. BeWo cells transfected 63-72 hours prior with 40 nM siRNA duplexes were dosed with either 5 nM $[^3]$H-$B_2$ or 10 nM $^{125}$I-transferrin (TF) for 4 min at 37 °C or 4 °C. Both actively internalized $B_2$ (minus passively absorbed $B_2$, A) and TF (B) are significantly reduced exclusively under DNM2 pool siRNA treatments. The results reflect the standard deviations of 4-6 separate experiments. Statistical significance between targeting siRNA and non-targeting treatments was defined using one-way ANOVA with Newman-Keuls multiple comparison test (*p < 0.05).
3.3.4 Extent of plasma membrane bound versus internalized ligand as a function of siRNA treatment

Dynamin 2 GTPase has been extensively characterized to function as a critical gate keeper of intracellular trafficking in that it is required in endosomal vesicle formation and release from the plasma and Golgi membranes (Maier et al., 1996; Hinshaw, 2000; McNiven et al., 2000; Dahan et al., 2005). Without functional dynamin 2, RME mechanisms such as clathrin-dependent RME are prevented from forming endosomal vesicles and thus unable to transport their cargo from the plasma membrane or Golgi membrane to various other destinations in the cell. Instead, ligand-bound receptors are restricted to these membranes. In light of the evidence of attenuated uptake for both B₂ and TF shown exclusively under silenced dynamin 2 treatments, we would expect a concomitant increase in ligand concentrations bound at the cell surface. To test this hypothesis we analyzed the extent of plasma membrane bound ligand detected after the 4 min uptake period, and compared this data to the extent of internalized ligand. Membrane bound and internalized ligands detected under the varying 40 nM siRNA treatments were normalized to non-targeting siRNA effects, and were expressed using equations 1 and 2 defined under methods and materials.

As expected, DNM2 pool siRNA treatments led to a more pronounced increase in the extent of bound B₂ at the plasma membrane, which coincided with a concomitant decrease in its internalization. Likewise, TF localized to the plasma membrane was enhanced to a greater extent under silenced dynamin 2 conditions. Under silenced dynamin 2 states, plasma membrane bound B₂ was shown to be ~ 150% higher than
bound ligand revealed for untreated cells (Figure 3.4A). In addition, internalized riboflavin reduced by $\sim 40\%$ compared to untreated conditions. Although to a lesser extent than that shown for riboflavin, membrane bound TF detected for cells treated with DNM2 pool siRNA increased $\sim 110\%$ over bound ligand detected in untreated cell populations (Figure 3.4B). Furthermore, TF uptake reduced $\sim 30\%$ under reduced dynamin 2 levels as compared to untreated conditions. Both B$_2$ and TF showed substantial localization at the plasma membrane with dynamin 2 silenced treatments when compared to the corresponding internalized ligand data. Both ligands revealed nearly 200% higher enrichment at the plasma membrane than within the cell. When the extent of bound over the extent of internalized ligand was expressed as a ratio, both riboflavin and transferrin were shown to be 3.1- and 2.0 fold higher than that defined for untreated conditions, respectively (Figure 3.4C and D).
Figure 3.4. Effect of siRNA treatments on the extent of plasma membrane bound ligand versus internalized ligand. BeWo cells transfected with 40 nM duplex siRNA were used in uptake assays (63-72 hours post-transfection) to examine the extent of bound versus internalized $[^3]$H-B$_2$ and $^{125}$I-TF. Data analyses involved equations 1 and 2 defined under methods for membrane associated and internalized B$_2$ (A) and TF (B). Ligand localization was further characterized for the overall extent of plasma membrane bound over internalized ligand. Riboflavin revealed nearly 3 fold higher localization at the plasma membrane under DNM2 pool siRNA treatments compared to untreated conditions (C). Likewise, TF resulted in ~2 fold higher enrichment at the membrane surface with DNM2 pool siRNA treatments compared to untreated conditions (D). These data represent the standard deviations of 3 to 8 separate experiments. Statistical significance between targeting and non-targeting effects on ligand trafficking was determined using one-way ANOVA with Newman-Keuls multiple comparison test (*$p < 0.05$, ***$p < 0.001$).
3.3.5 Effect of transiently transfected wild-type or dominant-negative dynamin 2 (K44A) expression constructs on the extent of bound and internalized ligands

A common approach in corroborating RNAi data involves the use of wild-type and dominant-negative expression vectors. We obtained the characterized wild-type dynamin 2 (DNM2<sup>WT</sup>) and GTPase-null dynamin 2 (DNM2<sup>K44A</sup>) expression constructs, the latter of which is unable to hydrolyze GTP and thus unable to pinch off nascently formed endocytic vesicles at the plasma membrane (Cao et al., 1998). BeWo cells were transiently transfected with 400 ng of plasmid DNA (wild-type dynamin 2 (DNM2<sup>WT</sup>), GTPase-null dynamin 2 (DNM2<sup>K44A</sup>), or the empty expression vector) in the presence or absence of 40 nM DNM2 pool siRNA according to the same methods used in siRNA transfections. Cells were used in uptake assays 64-72 hours post-transfection, at which time cells were dosed with either 5 nM [³H]-B<sub>2</sub> or 10 nM <sup>125</sup>I-TF for 4 min. As mentioned earlier, all internalized B<sub>2</sub> data generated at 37 °C was corrected for the passively absorbed component by subtracting the corresponding results obtained at 4 °C. When both B<sub>2</sub> and TF results were expressed as a ratio of plasma membrane bound over internalized, a strikingly similar trend was revealed (Figure 3.5). Both ligands were shown to be largely localized at the cell membrane, as opposed to intracellularly, under GTPase-null dynamin 2 alone and cotransfection conditions (i.e., DNM2<sup>K44A</sup> alone or DNM2 siRNA + DNM2<sup>K44A</sup>, respectively). Although not significant, plasma membrane bound B<sub>2</sub> under GTPase-null dynamin 2 conditions was shown to increase 1.5 fold and 1.7 fold over cells transfected with wild type dynamin 2 or the empty vector alone, respectively (figure 3.5A). The enhanced enrichment of B<sub>2</sub> to the membrane surface was
shown to be more pronounced under cotransfection conditions (i.e., DNM2 siRNA + DNM2$^{K44A}$), of which led to 1.8 fold, 2 fold and 1.4 fold higher membrane localization when compared to the effects of wild type, the empty vector, and the cotransfection treatment involving non-targeting siRNA and the empty vector, respectively. A similar, though more significant, trend was noted for the control ligand, transferrin. Transient transfections involving GTPase-null dynamin 2 revealed nearly 2 fold and 2.4 fold higher TF localization at the plasma membrane versus the effects of wild type dynamin 2 and empty vector treatments, respectively (figure 3.5B). Similar to B$_2$, the cotransfection condition led to a more pronounced enrichment of TF at the membrane surface that was 1.5 fold and nearly 3 fold higher than the effects of the negative control cotransfection treatment (i.e., NT siRNA + empty vector) and the empty vector, respectively. These results are in agreement with data from cells transfected with DNM2 pool siRNA alone, and provide additional evidence that actively internalized riboflavin requires the functional expression of the mechanoenzyme, dynamin 2, for its endocytic trafficking in human placental trophoblasts.
Figure 3.5. Effects of the over expression of wild type and GTPase-null dynamin 2 on intracellular and plasma membrane ligand localization. BeWo cells were transiently transfected with 400 ng each of either wild type dynamin 2 (DNM2WT), GTPase-null dynamin 2(DNM2K44A), or with a combination of DNM2K44A and 40 nM DNM2 pool siRNA. Sixty three to 72 hours post-transfection, cells were dosed with 5 nM [3H]-B2 or 10 nM 125I-TF for 4 min at 37 °C and examined for membrane associated and internalized ligands. Analysis of plasma membrane bound B2 over internalized B2 (A) revealed a trend in increased membrane bound ligand exclusively under single GTPase-null dynamin 2 transfection (DNM2K44A) and cotransfection (DNM2 siRNA + DNM2K44A) conditions. Similar results were exhibited by the control ligand, TF (B). The data represents the standard deviations of 3-4 separate experiments. The effects of dynamin 2 expression vectors or GTPase-null dynamin 2 cotransfections with DNM2 siRNA on ligand localization were defined through comparisons to single transfections with an empty vector or empty vector cotransfections with 40 nM non-targeting siRNA (NT siRNA), respectively. Statistical significance between experimental treatments and control conditions were determined using one-way ANOVA with Newman-Keuls multiple comparison test (*p < 0.05, **p < 0.01).
3.4 Discussion

A combinatorial approach involving RNAi and wild-type or dominant-negative plasmid transfections was carried out to delineate the involvement of the conserved endocytic mechanoenzyme, dynamin 2, in riboflavin absorption in BeWo cells. Collectively the RNAi and dynamin 2 expression construct data revealed that the endocytosis of riboflavin is regulated, in part, by the functional expression of dynamin 2 GTPase (Figures 3.1-5). In BeWo cells, the extent of dynamin 2 dependence in regulating cellular riboflavin levels was similar to that of the clathrin-dependent RME ligand, transferrin. Attenuated internalization of both B2 and TF (~ 60 and 70% of control conditions, respectively) correlated with ~ 80% reduced dynamin 2 levels. In addition, reductions in ligand uptake under these same conditions revealed a concomitant increase in B2 and TF binding along the cell surface. Furthermore, cotransfection results involving the GTPase-null dynamin 2 expression construct and DNM2 siRNA illustrated an amplified effect on the predominant ligand accumulation at the plasma membrane for both B2 and TF. Although the riboflavin receptor remains to be identified, this data provides the most substantial evidence to date supporting the existence of such a protein.

The fact that the reduced dynamin 2 protein expression does not reduce transferrin uptake more profoundly poses a concern. A common technique applied by other laboratories performing RNAi studies is to carry out two consecutive transfections (~ 24 hours apart) of cells in order to achieve maximal gene silencing and target protein knockdown. Such strategies have produced more substantial functional effects in other cell models, with HeLa cells being a gold standard system. For instance, Huang and
coworkers (Huang et al., 2004) revealed ~ 70-80% inhibition in transferrin absorption in HeLa cells that were transfected twice with siRNA targeting the clathrin heavy chain (i.e., critical protein subunit of the clathrin triskelion complex) or dynamin 2. In an effort to duplicate this approach in BeWo cells, two consecutive transfections were carried out using the DNM2 siRNA treatments. However, this strategy caused the cells to stop growing and cell morphology appeared to be compromised (data not shown). In another study (Soulet et al., 2005) the researchers performed a single siRNA transfection in HeLa cells using siRNA targeting sorting nexin 9 (SNX9), which is a protein that binds and regulates dynamin 2 activity and clathrin-mediated endocytic efficiency. They reported a 45% reduction in transferrin absorption, which is comparable to the effect we observed for this control ligand in BeWo cells treated with DNM2 siRNA (i.e., 30% reduced transferrin uptake). The close agreement between our results in BeWo and that of Soulet and coworkers’ studies in HeLa suggest our RNAi strategies are specific and effective in eliciting functional effects on the trafficking of the control ligand, transferrin.

In summary, the RNAi and dynamin 2 plasmid DNA transfection data provide definitive and direct evidence that a riboflavin receptor-mediated endocytic mechanism exists in human placental trophoblasts. Dynamin 2 GTPase is the first protein identified in humans to serve as a regulator of B₂ cellular entry through RME. Knowing that dynamin 2 is required for the majority of known endocytic pathways, and in light of the significant reduction in B₂ uptake under silenced dynamin 2 levels, we can estimate that the endocytosis of this critical vitamin accounts for up to 50% of the riboflavin-specific active absorption mechanisms in BeWo. Analogous to the folate and transferrin receptor
pathways, understanding B₂ endocytic internalization and trafficking provides a novel pathway that may be potentially exploited in targeted drug therapeutics and/or biomedical imaging (Li et al., 2003; Berry et al., 2004; Hattori and Maitani, 2004; Yoo and Park, 2004a).
CHAPTER 4

ELUCIDATING THE ROLE OF CAVEOLIN 1 IN THE RIBOFLAVIN ENDOCYTIC ITINERARY IN PLACENTAL TROPHOBLASTS

Abstract

Riboflavin (vitamin B₂) is an essential water soluble vitamin that is thoroughly established to be critical to cellular growth, function, and development. In its coenzyme forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), it is indispensable in a myriad of metabolic pathways. Despite its biochemical and physiological importance, the specific active absorption and trafficking mechanisms regulating B₂ cellular entry remain to be characterized. Recent reports suggest riboflavin is absorbed into human epithelia via a clathrin and dynamin 2 dependent endocytic process (Chapters 2 and 3). Considering dynamin 2’s indiscriminate promotion of distinct receptor-mediated endocytic mechanisms, riboflavin absorption may occur through alternative clathrin-independent endocytic pathways. The purpose of this study was to delineate the involvement of the clathrin-independent caveolae-mediated endocytic
mechanism in regulating B2 cellular entry into human placental trophoblasts (BeWo).

Endocytosed rhodamine-riboflavin was examined for colocalization with the immunofluorescence detected caveolae-associated protein, caveolin 1, using 3D confocal laser scanning microscopy. Although to a lesser extent than that for the control ligand, cholera toxin subunit B, ~11% of rhodamine-B2 was shown to distribute to caveolin 1-positive endosomes upon 10 min uptake. Results from this study suggest that B2 uptake is minimally regulated by caveolae-mediated endocytosis.
4.1 Introduction

Riboflavin (vitamin B$_2$) plays a vital role in normal cellular growth and maintenance. At the molecular level, B$_2$ serves as the precursor molecule to the coenzyme forms, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Both FAD and FMN have been extensively recognized for their critical function as coenzyme prosthetic groups of flavoproteins, which are indispensable macromolecules involved in a multitude of cellular redox reactions (Voet, 1995). Since humans are unable to synthesize B$_2$, they are restricted to obtaining this vitamin through their diet or through symbiotic microorganisms that naturally synthesize this vitamin, and are known to colonize the gut lumen. Despite its rare occurrence, B$_2$-deficiency has been documented in humans and is correlated with such factors as dietary deprivation or physiological and/or pathological conditions. Common manifestations resulting from B$_2$-deficient states in humans include growth retardation, neurodegeneration, skin lesions, and anemia (Cooperman, 1991). More severe cases have been documented with animals. Such studies have indicated a general sequence of symptoms including stunted growth, muscle weakness, and failure to thrive that leads to eventual death (Cooperman, 1991). Several pathological states have also been correlated with mammalian B$_2$ levels. In general, studies have shown B$_2$ to function as a protective factor in preventing cardiovascular disease in humans (Hultquist, 1993), cataract formation in animals (Wintrobe, 1994; Miyamota, 1998), and certain human cancers (Powers, 2003a). Despite extensive evidence suggesting the physiological and pathological importance of B$_2$ across different species, the factors regulating its cellular absorption and trafficking are poorly understood.
To date, the majority of documented studies defining the factors regulating B₂ absorption have been based on biochemical assays (Foraker et al., 2003). Combined, these studies have established several common regulatory parameters that are observed across divergent species, and include temperature dependence, Na⁺-independence, general energy dependence, active transport kinetics at physiological B₂ levels and passive diffusion kinetics above basal B₂ concentrations. These factors alone indicate the existence of a transport mechanism specific to this vitamin; however, they do not distinguish between a carrier-mediated or receptor-mediated process. More recent studies have provided evidence of microtubule-driven vesicle trafficking events, specific to receptor-medicated endocytosis (RME), being involved in the regulation of B₂ absorption by various human-derived cell lines (Wangensteen et al., 1996; Holladay, 1999; Huang and Swaan, 2000; Huang and Swaan, 2001). Additional support for a B₂-specific receptor-mediated mechanism has been established through 2D fluorescence microscopy and 3D confocal laser scanning microscopy-based assessments showing rhodamine-labeled B₂ localization to early endosomes of the clathrin-mediated endocytic (CME) pathway (Huang et al., 2003; D'Souza et al., 2006b). Such data was further corroborated by evidence of a time- and dose-dependent [³H]-B₂ accumulation to clathrin pathway specific endosomal populations that were isolated using sucrose density-based subcellular fractionation (Chapter 2) (D'Souza et al., 2006b). More recent unpublished studies aimed at elucidating the involvement of the major endocytic vesicle scission protein, dynamin 2 GTPase, have clearly demonstrated that ~ 50% of actively absorbed B₂ depends on the functional expression of this conserved protein (Chapter 3). Considering the pluripotent
nature of dynamin 2 on regulating the nascent formation and release of endocytic vesicles from the plasma membrane for different RME pathways, it becomes plausible that B2-RME may involve multiple, distinct endocytic mechanisms.

Caveolin 1 (CAV1) is thoroughly established to function in caveolar-mediated endocytosis (CvME) in mammalian cells (Pelkmans, 2001; Pelkmans et al., 2001; Pelkmans et al., 2002; Nabi and Le, 2003; Pelkmans et al., 2004; Pietiainen et al., 2004; Pelkmans et al., 2005; Pelkmans and Zerial, 2005; Tagawa et al., 2005). Specifically, CAV1 is a cytoplasmically oriented integral membrane protein known to bind cholesterol (Uittenbogaard and Smart, 2000) and associates with cholesterol- and sphingolipid-rich plasma membrane domains termed caveolae. CAV1 is one of three known caveolin isoforms that has been characterized to be critical in signal transduction pathways, membrane organization, and ligand bound receptor mediated trafficking in many divergent cell systems (Cheng et al., 2006). Some of the classical ligands that have been characterized to be absorbed and trafficked via this pathway include vitamin D, cholera toxin subunit B, and folate, to name a few (Parton, 1994; Mineo and Anderson, 1996; Norman et al., 2002; Singh et al., 2003; Huhtakangas et al., 2004; Hansen et al., 2005; Norman, 2006). Furthermore, caveolae-mediated endocytosis has been shown to be dependent on dynamin 2 expression (Yao et al., 2005), however it is distinct from the classical clathrin-dependent endocytic pathway (Cheng et al., 2006).

Although riboflavin absorption and its cellular distribution have been extensively shown to involve clathrin-mediated endocytosis (Huang and Swaan, 2000; Huang and Swaan, 2001; Huang et al., 2003; D'Souza et al., 2006b), the involvement of other
endocytic pathways remains to be elucidated. Caveole-mediated endocytosis is a candidate B₂-specific RME pathway known to be regulated by dynamin 2.

The objective of this study was to (a) evaluate whether B₂-specific RME involves the clathrin-independent caveolar-mediated internalization pathway in the human placental trophoblast cell model, BeWo, and if so, (b) to define the extent by which this alternative endocytic mechanism may regulate the active absorption of this vitamin. Studies involving fluorescent ligand absorption assays combined with indirect immunofluorescence detection of caveolin 1-positive endosomes were carried out to define the role of caveole-mediated endocytosis in riboflavin homeostasis.

4.2 Methods and materials

4.2.1 Cell culture

Human placental trophoblasts (BeWo) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a controlled atmosphere at 37 °C, under 5% CO₂. BeWo cells (passages 204-215) were cultured in F-12K growth medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 units/mL penicillin, and 100 µg/mL streptomycin.

4.2.2 Fluorescent ligand endocytosis assay and immunofluorescence staining

Cells were seeded 3-5 days prior (5 x 10³ cells/cm²) in collagen-coated BD Falcon culture slides (BD Biosciences, Bedford, MA). Following serum starvation, pulse-chase assays with either 500 nM rhodamine-riboflavin (Rd-RF) (Phelps et al., 2004) or 15 nM AlexaFluor®555-labeled cholera toxin subunit B (CTX, Invitrogen™, Molecular Probes™, Eugene, OR) were carried out according to established methods (Huang et al.,
Cells were immediately fixed with 4% paraformaldehyde, permeabilized, and blocked with bovine serum albumin prior to the immunolabeling of caveolin 1 with rabbit-anti-CAV1 (Sigma, Saint Louis, MO) for 1 hr at room temperature. Cells were thoroughly washed and probed with AlexaFluor®405-labeled goat-anti-rabbit IgG (Invitrogen™, Molecular Probes™, Eugene, OR). Immunofluorescence treatments were preserved using GelMount™ (Biomed Corp., Foster City, CA).

4.2.3 Fluorescence image acquisition, restoration, and colocalization analysis

Internalized fluorescent ligands and immunostained caveolin 1 were imaged using a Nikon Eclipse TE2000 E inverted confocal laser scanning microscope (Nikon Instruments Inc., Melville, NY) outfitted with fixed lasers for 405 nm and 543 nm. Three dimensional images were acquired using the following settings on Nikon EZ-C1 software (Gold version 2.3, Image Systems Inc., Columbia, MD): Nikon Plan Apo 60x A oil objective (1.4 numerical aperture), 3.6 μs scan dwell time, 512 x 512 pixel size, 0.30 μm z-step, and a 150 detector pinhole. Raw images were iteratively deconvolved using a calculated point spread function for individual channels and corrected for background noise using a combination of median filtration and setting threshold levels just above negative control treatments with either a non-reactive rhodamine derivative or the secondary AlexFluor®405 antibody alone. Restored images were analyzed for 3D colocalized fluorescence between ligands and caveolin 1 using Volocity, version 3.6 (Improvision Inc., Lexington, MA). The extent of colocalization between ligands and caveolin 1 were determined by calculating the percent of total overlap volume (μm³) over...
the corresponding total ligand volume. True colocalization between channels was defined using the statistical test, the Pearson’s correlation (Manders et al., 1993).

4.3 Results

4.3.1 Colocalization of rhodamine- riboflavin with the conserved endocytic coat protein, caveolin 1

The intracellular localization of B2 with respect to the caveolae-associated vesicle protein, caveolin 1 (CAV1), was examined using the characterized rhodamine-labeled riboflavin (Rd-RF) conjugate, which has been shown to exhibit nanomolar affinity characteristics similar to the natural ligand (Huang et al., 2003). Ligand internalization assays were carried out using subconfluent cells dosed with either 500 nM Rd-RF or 15 nM AlexaFluor®555-labeled cholera toxin subunit B (CTX) for 10 min prior to cell fixation. Fluorescently-tagged CTX is thoroughly characterized to be endocytosed through clathrin- and caveolae-dependent pathways across divergent cell models, and thus serves as a positive control ligand in these assays (Parton, 1994; Henley et al., 1998; Singh et al., 2003; Hansen et al., 2005; Yao et al., 2005). Internalized Rd-RF and CTX were examined for colocalized signals with immunostained CAV1 in BeWo cells using 3D confocal laser scanning microscopy. Fluorescence signals specific to Rd-RF were normalized to fluorescence intensities detected in parallel cells treated exclusively with a non-reactive rhodamine analogue, caboyxtetramethyrrhodamine-4-amine (CTMR4A), of which is a known by-product of the Rd-RF conjugation reaction (Phelps et al., 2004). Furthermore, AlexaFluor®405-labeled secondary antibody specificity to the CAV1 antibody was defined by omitting the primary antibody in parallel treatments. Signal-to-
noise ratios for cells treated with either CTMR4A or the fluorescent-tagged secondary antibody were substantially lower than cells treated exclusively with Rd-RF or the combined primary and secondary antibodies, respectively (data not shown). Both Rd-RF and CTX resulted in punctate perinuclear staining resembling endosomal organelle localization (Figure 4.1). In contrast to CTX, Rd-RF resulted in minimal overlap with CAV1.
Figure 4.1. Colocalization of rhodamine-riboflavin (Rd-RF) and cholera toxin subunit B (CTX) with the endosomal coat protein, caveolin 1 (CAV1). Rd-RF and AlexaFluor® 555-labeled CTX were examined for colocalization with the immunostained early endocytic protein marker, CAV1, after ligand internalization in BeWo cells for 10 min. Images represent orthogonal 3D profiles with the inset view defining the XY plane, and the outer panels reveal the YZ (right narrow panel) and XZ (top narrow panel) focal planes. Fluorescence signals for each channel were merged to reveal regions of colocalization (indicated by arrows and yellow regions) and overlaid with the corresponding differential interference contrast (DIC) image to define cell morphology (far right inset view). Scale bars (micrometers) are defined in the merged inset panels.
The extent of colocalization between ligands and CAV1 were defined as the percent of the total detected ligand volumes (cubic µm). The control ligand, CTX, revealed ~ 3.3 fold higher (39.16 ± 2.91%) colocalization with CAV1 compared to the signal overlap seen for Rd-RF and CAV1 (11.71 ± 0.76%) (figure 4.2). Overlapping fluorescence intensities between ligands and CAV1 were further examined for 3D shape similarities using the Pearson’s correlation statistical test (Manders et al., 1993). Specifically, this test is commonly used to reveal linear relationships existing between colocalized objects. Values > 0.0 define positive overlap between fluorescent channels. Both ligands revealed positive fluorescence signal overlap with CAV1, and thus further suggest ligand enrichment to caveolar endosomal populations. Collectively, these results in combination with the morphological assessments shown in figure 4.1 indicate significant CTX recruitment to CAV1-positive endosomes, which is in agreement with literature results (Parton, 1994; Singh et al., 2003; Hansen et al., 2005). In striking contrast, Rd-RF was shown to minimally localize with caveolae-associated vesicles. This data suggests that B2 internalization through the caveolae-mediated endocytic pathway occurs to a minor extent compared to that for CTX.
Figure 4.2. Quantitative evaluation of the 3D colocalized regions of Rd-RF and CTX with CAV1 endocytic protein marker. Overlapping (i.e., colocalized) volumes (cubic µm) for either Rd-RF or CTX with the caveolin 1 channel were expressed as a percentage of the total volume for each ligand. The Pearson’s correlation (PC) was chosen to define the likeness in 3D shapes between overlapping ligand and CAV1 channels. Data are expressed as the mean ± the standard error of the mean for three regions of interest.
4.4 Discussion

Recent reports by our laboratory revealed a clathrin-mediated endocytic component regulating riboflavin absorption and trafficking in divergent cell systems (Huang and Swaan, 2000; Huang and Swaan, 2001; Huang et al., 2003; D'Souza et al., 2006b). Furthermore, the functional expression of the vesicle scission protein, dynamin 2 GTPase, of which is well known to be critical in the early stages of endosome formation common to the majority of characterized endocytic pathways, has been shown to play an important role in regulating the internalization of B2 in human placental trophoblasts (Chapter 3). Considering the pluripotent nature of dynamin 2 on regulating different RME mechanisms, the question remains as to whether riboflavin is internalized by multiple endocytic pathways. In this study, our data reveals minimal involvement of the clathrin-independent caveolae-mediated endocytic pathway in regulating the cellular entry of B2.

Established fluorescent ligand endocytosis assays involving the characterized rhodamine-riboflavin conjugate were carried out to define the extent of colocalized signal intensities with the immunofluorescence detected caveolar endosome marker protein, caveolin 1. Three-dimensional confocal laser scanning microscopic analysis revealed ~11% colocalization between Rd-RF and CAV1 after 10 min uptake in the BeWo model. This degree of signal overlap was in dramatic contrast to that for the positive control ligand, cholera toxin subunit B, which revealed nearly 40% localization to caveolin 1 positive endosomes. Combined with prior reports by our laboratory, the active absorption of B2 in human epithelia is revealed to be regulated to a minor extent by the caveolar
mediated pathway, while the clathrin-dependent trafficking mechanism appears to be the dominant route of cellular entry.

The concept of multiple endocytic processes regulating the cellular entry of a single ligand is not unique to riboflavin. In fact, evidence of this phenomenon has been demonstrated for the tumor growth factor beta (TGF-β), interferon-gamma, and cholera toxin subunit B (Sadir et al., 2001; Di Guglielmo et al., 2003; Hansen et al., 2005). Considering the importance of maintaining cellular riboflavin levels required for normal growth and development, the existence of multiple active uptake mechanisms would provide additional controls to meet such nutritional demands in states of physiological distress. In the case for TGF-β, Di Guglielmo and colleagues demonstrated a biochemical feedback mechanism regulating its internalization via either the clathrin-mediated or caveolar-mediated pathways (Di Guglielmo et al., 2003). Specifically, their data revealed the absorption of TGF-β along the CME pathway correlated with a signal transduction response as defined by interactions with the Smad anchor for receptor activation (SARA) protein. In contrast, caveolae-mediated endocytosis (CvME) of TGF-β was coupled with the Smad7-Smurf2-dependent receptor degradation response and led to ubiquitin-dependent degradation of the TGF-β receptor. In this particular instance, the CvME mechanism appears to be involved in receptor degradation and ultimately receptor turnover, whereas the CME pathway functions in promoting signal transduction cascades. However, there are also reports that have shown the CME pathway to facilitate receptor degradation through trafficking to lysosomal organelles, as is documented for the epidermal growth factor (de Melker et al., 2001). In the case for riboflavin, the
involvement of two distinct RME pathways regulating its cellular entry may similarly reflect a homeostatic seesaw of molecular cascades resulting in either B$_2$-receptor activation coupled with increased vitamin absorption, or receptor degradation and reduced ligand uptake.

The involvement of multiple endocytic pathways regulating riboflavin internalization opens up novel drug targeting mechanisms that can bypass efflux transporters and thus may potentially improve drug bioavailability. Conceptually and empirically, such drug formulations have been demonstrated for the folate and transferrin RME pathways (Singh, 1999; Stephenson et al., 2003; Ghaghada et al., 2005; Hattori and Maitani, 2005; Soni et al., 2005; Lubgan et al., 2006; Singh et al., 2006; Suresh Reddy et al., 2006). Current studies in our laboratory are focused on defining the potential of exploiting the B$_2$-RME pathways for targeted chemotherapeutics using riboflavin-conjugated polymeric drug carriers. Furthermore, recent reports (Rao et al., 1999; Karande et al., 2001; Rao et al., 2006) revealing substantially elevated serum riboflavin binding protein (RfBP) levels in breast and liver cancer patients compared to healthy subjects proposes a potential targeted-chemotherapeutic niche for such B$_2$-conjugated formulations.

The purpose of this study was to elucidate whether alternative, clathrin-independent, endocytic mechanisms regulate B$_2$ cellular entry in the human placental trophoblast cell model, BeWo. The caveolae-mediated endocytic pathway was chosen as a candidate mechanism largely based on the extensive reported knowledge defining this major clathrin-independent route for ligand internalization. Caveolin 1, an endosomal
protein marker specific to caveolae-dependent endocytosis, served as the indicator of this pathway in 3D fluorescence colocalization assessments of the intracellular distribution of rhodamine-riboflavin. Although to a minor extent compared to the positive control ligand, CTX, B$_2$ absorption was shown to involve CvME in BeWo. Ideally, such data would be more informative compared to the extent of colocalization between B$_2$ and clathrin-positive endosomes occurring after the same uptake period. Such studies would further define the overall importance of the CME and CvME pathways in regulating B$_2$ cellular entry. Ongoing studies are addressing this concern in the effort to more clearly define the B$_2$-RME trafficking itinerary in placental trophoblasts.
CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

5.1 Summary

The objective of this thesis was to characterize the receptor-mediated endocytic mechanism(s) (RME) regulating riboflavin absorption and trafficking in human derived placental trophoblasts and intestinal cell systems, BeWo and Caco-2, respectively. Results from these studies record for the first time the temporal and spatial accumulation of riboflavin to clathrin-associated early endosomal organelles that is suggested to be negatively regulated by increasing cAMP levels (Chapter 2). The extent by which B\textsubscript{2} internalization relies on RME in BeWo cells was further demonstrated (~ 50%) as a function of the expression level of the master endocytic vesicle scission enzyme, dynamin 2 GTPase (Chapter 3). Finally, preliminary studies examining whether clathrin-
independent RME processes are regulating the active internalization of B₂ revealed a minor involvement of the caveolae-mediated endocytosis pathway as demonstrated through 3D fluorescence colocalization between rhodamine-riboflavin and the caveolar coat protein, caveolin 1 (Chapter 4).

Classical clathrin-dependent receptor mediated endocytosis (CME) is characterized by sequential orchestrated signaling cascades involving protein-protein and protein-lipid interactions (Figure 5.1) (Merrifield et al., 2005; Perrais and Merrifield, 2005). Initial stages at the plasma membrane involve the nucleation of a clathrin-coated pit (CCP), which is suggested to occur when the adaptor protein 2 (AP-2) binds to phosphoinositol bisphosphate (PIP2). This interaction coincides with the recruitment of clathrin and ligand-bound receptors through AP-2, and the consequential formation of a CCP. The CCP is anchored at the plasma membrane by cytoskeletal microfilaments and assessor proteins. Subsequent CCP growth and invagination events are then driven by a consortium of proteins, including the vesicular scission enzyme, dynamin 2 GTPase. Dynamin proteins polymerize to form a collar around the invaginated CCP neck, and through GTP hydrolysis, the dynamin collar drives the membrane budding and release of the vesicle from the plasma membrane into the cytosol. This nascently formed vesicle is termed an early endosome, and shortly after its release, the clathrin coat also detaches to repeat the cycle of CCP formation along the cytosolic face of the plasma membrane. Endosomally associated Rab GTPases, such as Rab5, then function in trafficking early endosome cargo to various destinations in the cell, including the Golgi, mitochondria, and lysosomes (Rhodaman, 2000; Qualmann and Kessels, 2002; Smythe, 2002).
**Figure 5.1. Initial stages regulating clathrin-dependent receptor-mediated endocytosis.**

(i.) Ligand-bound receptors expressed along the extracellular surface of epithelia are recruited by AP-2 and concentrated to clathrin coated pits (CCP) that become stabilized by microfilaments and accessory proteins. (ii.) Consequently, CCP growth and vesicle tubulation results through a consortium of protein-protein interactions, including the formation of a dynamin 2 collar at the CCP neck. (iii.) Through dynamin 2 dependent GTP-hydrolysis, the invaginated CCP is released from the plasma membrane, which is characterized by the association of the endosome trafficking protein, Rab5 GTPase, and is defined as an early endosome. (iv.) Almost immediately upon its release, the early endosome is stripped of its clathrin coat, allowing clathrin to return to the plasma membrane to repeat the ligand recruitment cycle of RME and the ligand-bound receptor laden early endosome to continue to traffic along the CME pathway.
In light of recent reports by our laboratory suggesting riboflavin absorption and trafficking is governed, in part, by clathrin-mediated endocytosis (Huang and Swaan, 2000; Huang and Swaan, 2001; Huang et al., 2003; D'Souza et al., 2006a; D'Souza et al., 2006b; Mason et al., 2006), and with the knowledge of the sequence of events regulating ligand trafficking along this pathway, riboflavin absorption and subcellular distribution is predicted to follow the dynamic and ordered trafficking itinerary specific to this mechanism. To test this hypothesis, a dual approach involving density gradient subcellular fractionation and 3D fluorescence confocal laser scanning microscopy was carried out to delineate the intracellular trafficking route specific to riboflavin in human intestinal (Caco-2) and placental trophoblast (BeWo) cells (Chapter 2). Analogous to the positive endocytic protein, transferrin, absorbed riboflavin was shown to largely accumulate to clathrin-associated early endosomes in a time- and dose-dependent manner in both cell models. Maximal B\textsubscript{2} accumulation to these endosomal populations was revealed to occur at a faster rate in Caco-2 (within 30 min) as compared to BeWo (within 60 min). However, a riboflavin dose-dependent effect on endosomal enrichment was shown to be dramatically more pronounced in the placental trophoblasts compared to the enterocytes. Although to a lesser extent, internalized riboflavin was also revealed to concentrate to Golgi and mitochondrial organelles in a time and ligand-dose dependent manner. This is the first report of riboflavin enrichment to Golgi and may suggest a possible recycling mechanism that is reminiscent of that reported for transferrin (Yamashiro et al., 1984; Woods et al., 1986). Furthermore, in agreement with the existence of a B\textsubscript{2}-specific receptor mediated endocytic mechanism, we revealed strict
homeostatic regulation of cellular riboflavin levels by cAMP. Collectively, these results, generated in divergent cell models, demonstrate a dynamic and ordered B₂ absorption and trafficking process consistent with the clathrin-dependent RME pathway. A variation in vitamin internalization rates and concomitant fortification to endosomal populations appears to reflect distinctive nutritional requirements that are met in a cell lineage-dependent manner.

Cumulative evidence has been shown to justify the clathrin-dependent B₂-RME process in human epithelia. Prior to the data presented in this thesis, no direct functional evidence had been reported regarding critical known human protein regulators governing riboflavin endocytosis. A strong candidate protein is Dynamin 2 GTPase. This ubiquitously expressed enzyme has been extensively documented to regulate the invagination and vesicle scission events occurring in classical endocytic pathways including clathrin-dependent and independent mechanisms. Consequently, dynamin 2 has been termed the master regulator of endocytic membrane trafficking events along the plasma membrane (Conner and Schmid, 2003). The potential involvement of dynamin 2 in the B₂-RME process was examined using the placental trophoblast model (Chapter 3). BeWo was chosen for these studies largely based on riboflavin’s substantial enrichment to clathrin-associated endosomes of this cell system as shown in Chapter 2, and for its higher affinity for B₂ compared to the intestinal cell model, Caco-2 (Huang and Swaan, 2001). Approaches leading to significantly reduced dynamin 2 expression (i.e., duplex siRNA-based RNAi methods) or the over expression of nonfunctional dynamin 2 (i.e., GTPase null dynamin 2K44A) were carried out in BeWo cells to define the extent by which
actively absorbed $B_2$ relies on RME. Similar to the positive control, transferrin, actively internalized $B_2$ was substantially reduced by $\sim 50\%$ exclusively under silenced dynamin 2 conditions. Furthermore, such reduced uptake correlated with a concomitant increase ($\sim 2$ fold higher than absorbed $B_2$) in $B_2$ localization at the cell surface. Similar ligand distribution profiles were shown under GTPase-null dynamin 2 conditions. In addition, a more pronounced $B_2$ localization at the cell surface was noted with cotransfection treatments involving both dynamin 2 silencing siRNA and the over expression of the GTPase-null protein, thus further suggesting the importance of this endocytic protein regulator in maintaining cellular $B_2$ levels. This study is the first to report the direct functional involvement of the conserved endocytic scission protein, dynamin 2 GTPase, in regulating riboflavin absorption in human epithelia. In addition, these studies demonstrate the general extent ($\sim 50\%$) by which riboflavin depends on RME in human placental trophoblasts.

Dynamin 2 GTPase is known to indiscriminately function in the vesicular budding and release of endosomes for multiple and distinct RME pathways. Therefore, if $B_2$ absorption is dependent on the functional expression of dynamin 2 as demonstrated in Chapter 3, there is a possibility that alternative clathrin-independent RME mechanisms may be regulating the cellular uptake of this vitamin. One such candidate pathway is the caveolae-mediated mechanism (CvME) (Nichols, 2002; Le and Nabi, 2003; Venkatesan et al., 2003). Like CME, CvME depends on functional dynamin 2 GTPase (Henley et al., 1998; Shajahan et al., 2004) to allow for the release of ligand-bound receptor laden vesicles from the plasma membrane. Caveolin 1 (CAV1) is a critical coat protein
characteristic of caveolae endosomes and is required in caveolar-mediated endocytosis (Nichols, 2003). In addition, CAV1 has been reported to interact directly with dynamin 2 (Yao et al., 2005). To demonstrate whether B₂ uptake involves CvME, BeWo cells were examined for colocalization between internalized rhodamine-riboflavin and fluorescently immunostained CAV1 using 3D fluorescence confocal laser scanning microscopy. Results from these studies revealed minimal colocalization between B₂ and the caveolae-associated endosomal marker, suggesting B₂ absorption and trafficking is regulated by CvME to a minor extent.

5.2 Conclusions: the B₂-RME model revised

For the past several decades, various studies have been carried out to elucidate the mechanisms regulating vitamin B₂ absorption in humans. From these studies, a set of B₂-specific parameters regulating its uptake kinetics have been generated and shown to be conserved across divergent mammalian cell models and tissues. These salient features include temperature-, energy-, and pH- dependence, active uptake and passive diffusion kinetics at low nM and above basal vitamin concentrations, respectively, and a general involvement of Ca²⁺-calmodulin, protein kinase A- and G- pathways (Huang and Swaan, 2000; Huang and Swaan, 2001; Foraker et al., 2003). In addition, 2D fluorescence microscopy studies analyzing the localization of absorbed rhodamine-labeled riboflavin in human placental trophoblasts suggested the involvement of the clathrin-mediated RME pathway in regulating cellular B₂ levels (Huang et al., 2003). In further agreement of a B₂ endocytic process, indirect biochemical-based modulator studies revealed riboflavin absorption and trafficking to be dependent on cytoskeletal scaffolds and associated motor
proteins common in microtubule and actin-dependent vesicle transport dynamics (D'Souza et al., 2006a). More recently, studies examining the effects of antibodies specific to the secreted avian-derived riboflavin binding protein (RfBP), of which is known to selectively bind extracellular B<sub>2</sub> and facilitate its cellular entry in oviparous species (Zheng et al., 1988; White et al., 1992; Wasylewski, 2004), was shown to dramatically reduce riboflavin absorption and trafficking in an RME dependent manner in human placental trophoblasts (Mason et al., 2006). Thus, analogous to the ligand sequestering functions of the characterized molecules transferrin and intrinsic factor, of which are known to regulate cellular iron and B<sub>12</sub> levels (Birn et al., 1997; Fyfe et al., 2004), respectively, the involvement of a soluble human RfBP protein is suggested in regulating B<sub>2</sub>-RME. Collectively, these studies indirectly indicate riboflavin absorption and trafficking in human epithelia is regulated, at least in part, by the classical CME pathway.

A major weakness to this B<sub>2</sub>-RME model is the lack of direct evidence identifying specific cellular proteins facilitating this vitamin’s uptake mechanism in humans. Although recent reports suggest the existence of a soluble human riboflavin binding protein, the direct identity of such a molecule remains to be established. Several laboratories, including our own, have attempted to isolate such a protein using various techniques including size-exclusion- and riboflavin-coupled affinity-chromatography, and riboflavin based photo-affinity labeling reactions. However, these studies were unsuccessful due to non-specific selection and labeling of ubiquitous flavoprotein molecules endogenous to all studied cell models and tissues. The aims in this thesis
involved alternative approaches to (1) resolve the subcellular distribution of absorbed riboflavin through subcellular fractionation and fluorescence 3D confocal laser scanning microscopy, (2) directly identify the functional involvement of the conserved pluripotent endocytic effector protein, dynamin 2 GTPase, using RNAi and dominant negative protein expression constructs, and (3) to determine the involvement of the clathrin-independent caveolae-mediated RME pathway, through 3D fluorescence colocalization analysis between internalized rhodamine-riboflavin and the immunostained caveolae-associated endosome marker, caveolin 1. Results from these studies provide additional justification of the clathrin-dependent B2-RME model, and further demonstrate for the first time the functional requirement of human dynamin 2 GTPase in this process (Figure 5.2).
Figure 5.2. The B₂-RME model revised. To date, the cellular entry of riboflavin along human epithelia has been definitively shown to occur through passive diffusion (A) and receptor-mediated endocytosis (B). In the case of B₂-specific RME, riboflavin is initially suggested to be bound by a putative human analog of extracellular avian-RfBP, of which is believed to aid in the recruitment of B₂ to its plasma membrane associated receptor. The ligand bound receptor complex is then sequestered to clathrin coated pits (CCP) (1). Through the sequential and concerted functions resulting from various protein-protein interactions including the vesicular scission action of dynamin 2 GTPase, this CCP becomes invaginated and is pinched off from the plasma membrane to form an early endosome (EE) (2). Through the functions of the associated Rab5 GTPase, the EE fuses with other early endosomes to form a late sorting or recycling endosome (LE) that characteristically has a lower pH than that of EE (3). At this point, the B₂-bound receptor complex may uncouple to generate free B₂ within the acidic LE. Similar to the transferrin-RME pathway, the unbound B₂-receptor and RfBP would then be free to recycle back to the apical membrane to recruit additional extracellular riboflavin (4a). Alternative destinations for trafficking of the receptor and RfBP molecules, in the absence or presence of B₂, from the LE are suggested to include the Golgi (4b), the basolateral membrane domain or systemic circulation through transcytosis (4c), lysosomes (L) for degradation (4d), or the mitochondria (4e). The nucleus (N) and endoplasmic reticulum (ER) are shown as points of reference.
Despite the extensive evidence indicating the involvement of the CME pathway in B2 cellular entry in human epithelia, several critical questions remain to be addressed at the molecular level. The identity of the human analogs for a riboflavin binding protein and the proposed B2-receptor remain to be established. In addition, earlier studies largely suggested a riboflavin-specific transporter was responsible for this vitamin’s active absorption in humans as reviewed by Foraker and coworkers (Foraker et al., 2003); yet, such a protein has not been definitively isolated or identified. Data presented in this thesis combined with earlier reports by our laboratory definitively justify the clathrin-dependent B2-RME model; however, these results don’t exclude the possibility of a transporter also being involved in regulating cellular B2 levels. To date, the involvement of a transporter in B2 absorption remains to be defined in humans.

5.3 Future directions: exploring the potential application of the B2-RME mechanism in targeted medicinal therapeutics

Some of the major obstacles in drug therapy include low permeability of hydrophilic drugs and/or macromolecules across cellular membranes, reduced cellular retention due to ubiquitously expressed efflux transporters, metabolic degradation by omnipresent enzymes, low mean residence time due to the reticuloendothelial system, and underlying genetic aberrations and/or pathological states exhibited by patients. All these factors combined drastically reduce drug bioavailability, and in certain instances increase drug-related toxicity. One approach to circumvent several of these issues has involved the packaging of biotherapeutics into ligand-targeted drug delivery systems.
Traditionally, fabrication of these devices has involved covalently conjugating natural biological ligands to lipid- or polymer-based nanoparticles that are loaded with various therapeutic entities. Such drug carriers bind to specific receptors along the cellular plasma membrane and then gain entry through endocytosis.

Ligand-targeted DDS technology has proven successful for targeting such natural ligand-receptor-mediated endocytic (RME) pathways as that for transferrin and folate. Kobayashi and coworkers (Kobayashi et al., 2007) demonstrated that drug efflux by P-gp is more effectively prevented in multidrug resistance (MDR) cancer cells when chemotherapeutics (e.g., doxorubicin) are delivered via liposomes targeted to transferrin receptors, as compared to untargeted liposome formulations. In another study by Kim and colleagues (Kim et al., 2006), reports revealed substantial enhancements in gene transfection efficiencies and gene silencing of GFP (green fluorescent protein) in the human epidermal carcinoma cell model, KB, upon transfecting cells with poly electrolyte DDS conjugated with folate. Specifically, their results demonstrated that such ligand-targeted gene silencing formulations’ effects could be achieved using significantly less short-interfering RNA (siRNA). In addition, these researchers demonstrated the entrapment of gene-silencing therapeutics in folate-tagged DDS enhanced the extent of cellular entry, increased siRNA stability against nuclease degradation, and further enhanced cell targeting specificity through the endogenous folate-specific RME pathway. Based on our current understanding of the riboflavin-specific RME pathway in epithelial cells, it would be interesting to test whether drug delivery systems targeting this pathway reveal similar advantages to gene and chemotherapeutic formulations as those shown for
transferrin receptor- and folate receptor-targeted DDS. Along with establishing a molecular map of B2 transport, metabolism and utilization in cellular growth and development, understanding the absorption and intracellular trafficking of riboflavin in human epithelia opens new opportunities for scientists to utilize this pathway for targeted drug delivery.
BIBLIOGRAPHY


