Recognition, Cointernalization, and Recycling of an Avian Riboflavin Carrier Protein in Human Placental Trophoblasts

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ABSTRACT

Absorption of riboflavin (RF) across membrane barriers is essential to cellular oxidation reduction processes. Riboflavin carrier protein (RCP), a 37-kDa secretory protein, is proposed to play an important role in RF absorption, although information on the mammalian ortholog remains unclear. This study alludes to the existence of a mammalian RF carrier protein and further characterizes its carrier role and fate using avian RCP in human placental trophoblast (BeWo), another mammalian cell line, monkey kidney (COS-1), and the avian control, chicken hepatic (LMH/2A) cells. The presence of RCP and its involvement in RF internalization was analyzed by immunofluorescence and immunobinding assays using chicken RCP (cRCP) antibodies. In the presence of anti-cRCP, cellular RF uptake is significantly decreased (5% of control) in BeWo cells. Kinetic analyses of intracellular accumulation of $^{125}$I-cRCP revealed a $J_{\text{max}}$ and $K_m$ of 28.56 ± 2.70 pmol/mg protein/min and 142.43 ± 82.16 nM, respectively, in BeWo cells and 75.14 ± 7.6 pmol/mg protein/min and 104.37 ± 23.96 nM in the species-specific control, LMH/2A cells. Subcellular fractionation studies revealed colocalization of both radiolabeled RF and cRCP within endosomal and lysosomal fractions, further elucidating RCP’s role in trafficking RF through the cell. Following intracellular release of RF from the carrier complex, the protein is either subject to lysosomal breakdown or is conserved via recycling mechanisms for continued RF sequestration and uptake. In summary, mammalian placental trophoblasts exhibit specific carrier protein dependence that sequesters and essentially mediates RF internalization via the proposed receptor-mediated endocytic pathway.

Riboflavin (vitamin B$_2$, RF), an essential micronutrient, exerts its role as coenzymic FMN and FAD via sequential redox reactions within the cell that drives intermediary metabolism of carbohydrates, amino acids, and lipids. Given the fundamental role of this water-soluble vitamin in cellular metabolism, its absorption into the cell solely from dietary sources has generated considerable interest. Previously, our laboratory identified receptor-mediated endocytic events in RF internalization in human intestinal and placental cells (Huang and Swaan, 2000, 2001). Trafficking upon entry into the cell has been well characterized and visualized using biochemical and microscopic approaches (Huang et al., 2003). However, the identity of this vitamin transport system specific to ligand recognition at the cell surface, its binding, and subsequent internalization all remain undefined.

Riboflavin binds nonspecifically to plasma proteins (albumin, globulins, and fibrinogen) (Combs, 1998), but analogous to other vitamins, such as retinol, vitamin D, and folate (Corrocher et al., 1991; Gomme and Bertolini, 2004; Zanotti and Berni, 2004), it exhibits specific binding to riboflavin carrier proteins (RCPs). Specific RCPs have been identified in the plasma of the laying hen, cow, mice, rats, spadefoot toad, turtle, bonnet monkey, and human umbilical cord serum (Foraker et al., 2003). However, to date, the molecular identity of this carrier protein remains undetermined. The increased gestational need for RF is pivotal to embryonic growth and development as evaluated in avian (Clagett, 1971) and rodent (Natraj et al., 1987) models and stipulates the pregnancy-specific role of hormonally regulated RCPs in RF delivery to the fetus (Murthy and Adiga, 1978a,b; Visweswariah and Adiga, 1988).

Avian riboflavin carrier protein [chicken RCP (cRCP)], the first RCP to be isolated and characterized, is an estrogen-inducible phosphoglycoprotein (37 kDa) that regulates oocyte transport of scavenged RF (Rhodes et al., 1959). Immunolog-
of RCPs maintain a degree of interspecies homology since several conformation-dependent monoclonal antibodies raised against the avian protein have been found to cross-react with simian (Visveswariah et al., 1987), rodent (Subramanian and Adiga, 1996), and human RCPs (Prasad et al., 1992; Subramanian and Adiga, 1999). Given this cross-reactivity and the mammalian taxonomy of this evolutionarily conserved vitamin carrier (Muniyappa and Adiga, 1980a; MacLachlan et al., 1993), the existence and role of a human RCP in mediating RF binding and translocation across the fetoplacental membrane barrier seems reasonable.

The working hypothesis describing cellular internalization of RF proposes its sequestration by RCP, recognition by membrane receptors, and uptake via clathrin-coated pits (MacLachlan et al., 1994; Huang et al., 2003). Attempts to identify cRCP receptors have revealed Ca$^{2+}$-dependent binding to members of the low-density lipoprotein family of receptors in association with vitellogenin, a broad-specificity carrier (Adiga et al., 1997). The present study uses mammalian cell lines (BeWo and COS-1) in parallel with avian controls (LMH/2A) to better understand the involvement of RCP in receptor-mediated RF internalization and its fate thereafter. Our combined data demonstrate that RCP translocates across cellular barriers, bringing with it bound ligand (RF) before recycling back to the extracellular milieu. Understanding this system may serve as a novel platform that could be targeted clinically toward cells with enhanced nutritional requirements for RF, such as the fetus and breast tumors.

Materials and Methods

Cell Culture. The BeWo, COS-1, and LMH/2A cell lines, obtained from American Type Culture Collection (Manassas, VA), were routinely maintained in a controlled and humidified atmosphere at 37°C under 5% CO$_2$. BeWo and COS-1 cells were cultured in F-12K and DMEM, respectively (Invitrogen Life Technologies, Carlsbad, CA), whereas LMH/2A cells were maintained in prepared Waymouth MB 752/1 medium (Sigma, St. Louis, MO). All media were supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. For uptake studies, cells were seeded in Costar 24-well plates (Fisher Scientific, Pittsburgh, PA) at a density of 1.0 to 1.5 × 10$^5$ cells/cm$^2$ and used 2 to 5 days after seeding.

Inhibition Assays. BeWo cells were incubated with 10 nM riboflavin-[3H]G (25 Ci/mmol; Sigma), and [3H]RF uptake was compared with accumulation of a nonspecific ligand (folic acid (FA)) in the presence of unlabeled RF (1 μM), bovine serum albumin (BSA; 30 μM), protein A-purified cRCP antisera (20–200 μg/ml), and preimmune rabbit sera (200 μg/ml). All solutions were prepared in Hanks’ balanced salt solution, pH 7.4, containing 25 mM glucose and 10 mM HEPES. After incubation, cells were processed as described previously (Huang and Swaan, 2001). In brief, cells were washed using ice-cold Dulbecco’s phosphate-buffered saline, pH 3.0, and lysed, and intracellular accumulation of [3H]RF was determined using a liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Results were normalized to total protein content determined by the Bradford assay (Bio-Rad, Hercules, CA).

Binding of [3H]RF to anti-cRCP was determined following a 10-min incubation at 37°C using BSA and cRCP as controls. Ligand-associated protein was captured using hydroxyapatite, whereas unbound [3H]RF was recovered in the supernatant after centrifugation at 10,000 rpm for 2 min. The hydroxyapatite pellet was washed with Tris buffer, pH 7.2, and bound radiolabel-associated protein was released using ethanol following centrifugation at 14,000 rpm for 3 min. Results were expressed as percentage total radioactivity.

Expression and Binding of Chicken Riboflavin Carrier Protein in Transfected Cells. A cDNA clone of cRCP, in pBluescript II KS+ ligated via NotI and EcoRI sites, was obtained from the Roslin Institute (Edinburgh, UK). The cRCP cDNA was extracted with HindIII and BamHI ligation sites via the forward primer, AAAAACTTATGCTGAGTTTGGATCAG, and the reverse primers, AAGGATCCATCTTCCTCCTCCCTCCTTC (with stop codon) and AAGGATCCATCTTCCTCCTCCTCCCTCCTCTTCC (without stop codon). Resulting cDNAs were ligated into pEGFP-N2 vectors (Clontech, Mountain View, CA) and sequenced on the DNA 3700 analyzer at the Plant-Microbe Genomics Facility at Ohio State University (Columbus, OH). For COS-1 transfection with cRCP, Lipofectamine 2000 and Opti-MEM (Invitrogen Life Technologies) were used as per the supplier’s protocol.

Medium binding assays were developed to detect the presence of RF-specific binding proteins secreted by transfected COS-1 cells into media. After 12- to 48-h incubations, medium was removed and incubated with 5 mg/ml activated charcoal under agitation at 4°C for 16 to 24 h. Samples were centrifuged at 12,000g, and supernatant was filtered using a 0.22-μm syringe filter. Cleared sample was then mixed 1:1 with 20 nM [3H]RF with or without 10 μM nonradiolabeled RF (to determine nonspecific binding) at 4°C overnight. All samples were processed using hydroxyapatite. Whole-cell binding was carried out similarly to uptake studies, with the exception that incubation with [3H]RF was for 2 h instead of 10 min.

Immunofluorescence Microscopy. BeWo and COS-1 cells transfected with cRCP were grown on four-well culture slides (Becton Dickinson, Bedford, MA) and processed for immunostaining as described previously (Huang et al., 2003). In brief, cells were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. To evaluate intracellular localization of cRCP, control cells were run in parallel under nonpermeabilizing conditions. Cells were then labeled with rabbit anti-cRCP antibody (1:250) at room temperature for 1 h and then incubated with Alexa Fluor 546-labeled goat anti-rabbit antibody (1:200) for 1 h at room temperature. Cells were counterstained with DAPI, washed, and mounted in SlowFade reagent. Images were captured with an RT SPOT CCD camera and software (National Diagnostics, Atlanta, GA) using the Nikon Eclipse 800 fluorescence microscope (Nikon, Melville, NY) equipped with a 40× objective and fluorescein isothiocyanate-HQY (λ$_{ex}$, 460–500 nm; λ$_{em}$, 510–650 nm; dichroic splitter, 505 nm), tetramethylrhodamine B isothiocyanate-HQY (λ$_{ex}$, 530–550 nm; λ$_{em}$, 590–650 nm; dichroic splitter, 565 nm), and UV-2E/C DAPI (λ$_{ex}$, 330–380 nm; λ$_{em}$, 435–458 nm; dichroic splitter, 400 nm) filter sets (Chroma, Rockingham, VT). Composite images were colored and assembled in Adobe Photoshop 6.0 (Adobe Systems Inc., Mountain View, CA).

Intracellular Uptake Kinetics of Iodinated-Chicken Riboflavin Carrier Protein. The apo form of cRCP (Sigma) 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), and $^{125}$I incorporation was assessed by gel electrophoresis and autoradiography. The specific activity of the $^{125}$I-cRCP was ~6700 cpm/pmol.

Cellular uptake of $^{125}$I-cRCP in confluent BeWo and LMH/2A cells was determined in the presence of 0 to 1 μM unlabeled cRCP and 2 to 25 μM monensin at 37°C. After 20 min, cells were processed as described above. Kinetic parameters were determined by nonlinear regression analysis using the following equation (Prism 4.0; GraphPad Software Inc., San Diego, CA):

$$V = \frac{V_{max}}{K_m + [S]} + K_d \times [S]$$

where $V$ represents the velocity of carrier-mediated uptake, $V_{max}$ represents the maximal transport capacity, $[S]$ represents the concentration of substrate, and $K_m$ and $K_d$ are the Michaelis and mass transfer constants, respectively.
centration of cRCP, \( K_a \) represents the Michaelis-Menten constant, and \( k_r \) represents the passive diffusion coefficient.

For recycling of cRCP, BeWo cells were loaded with 5 nM \( ^{125}\text{I}-\text{cRCP} \) for 20 min at 37°C. After internalization, cells were washed three times with Dulbecco’s phosphate-buffered saline, pH 7.4 and 3.0, to remove nonspecific and membrane-bound radioactivity. Fresh medium containing unlabeled cRCP was added to each well and maintained at 4, 16, or 37°C. Aliquots from the extracellular media were taken at regular intervals up to 1 h, and replacement buffer was added. After an hour, cells were washed thoroughly, lysed, and processed as described above to determine intracellular cRCP accumulation. Recycling \( t_{1/2} \) was determined using first-order kinetics (eq. 2; Prism 4.0):

\[
t_{1/2} = \frac{0.693}{k}
\]

where \( k \) is the recycling rate constant.

**Ligand Uptake and Subcellular Fractionation.** Cell monolayers were dosed with 10 nM each of \( ^{3}\text{H}\)riboflavin (Sigma) and \( ^{125}\text{I}-\text{cRCP} \) in Hanks’ balanced salt solution, pH 7.4, containing 25 mM glucose and 10 mM HEPES at 37°C for 2 h. After incubation, cells were washed thoroughly and pooled in homogenization buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, and protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN). Cells were allowed to swell for 15 min prior to lysis through a 25-gauge 5/8 hypodermic needle and then centrifuged at 600 \( g \) for 5 min at 4°C to yield a nuclear pellet and postnuclear supernatant.

For isolation of endosomes and lysosomes, the fractions were loaded on a discontinuous sucrose gradient (0.8–2.0 M), subjected to 25 min at 4°C to yield a nuclear pellet and postnuclear supernatant.

**Results**

**Presence of Specific RCP Involved in Riboflavin Uptake.** The presence of a mammalian carrier protein that facilitates RF uptake across membrane barriers has been alluded to previously (Merrill et al., 1979; Muniyappa and Adiga, 1980a; Visweswariah and Adiga, 1987a,b; Natraj et al., 1988), although its identity remains inconclusive. Given the suggested interspecies homology of this binding protein, human placental trophoblasts (BeWo) were treated with rabbit anti-chicken riboflavin carrier protein (anti-cRCP) to evaluate incidence and dependence of RF internalization on a specific and homologous protein recognized by the antisera. Treatment of the cells with anti-cRCP revealed a significant reduction in placental RF uptake (~95%) that occurred even at the lowest concentration of antibody (Fig. 1A). This indicates that RF internalization may depend on a specific mammalian protein that shares homology with its avian counterpart but is possibly poorly expressed and tightly regulated to influence ligand uptake in human trophoblasts. These findings were supported by visualization of anti-cRCP-immuno-reactive protein(s) detected only under permeabilizing conditions within the cellular compartment (Fig. 1D). Failure to detect signal under nonpermeabilizing conditions (Fig. 1D), together with the inhibition of RF uptake (Fig. 1A), suggests that mammalian cRCP is probably a secreted protein that is released in response to a ligand-triggered stimulus. Specificity of this inhibition with anti-cRCP was established using preimmune rabbit IgG and BSA as controls (Fig. 1A), whereas ligand specificity was confirmed by the limited inhibitory effect of anti-cRCP directly on RF uptake (Fig. 1C), with undetectable changes in intracellular folate accumulation (Fig. 1B). Unlike RF, the large excess of unlabeled folate showed submaximal inhibition of the labeled ligand, possibly due to the presence of multiple components that contribute to its internalization, namely the high-capacity folate carrier and high-affinity folate binding protein, respectively (Sirotnak and Tolner, 1999).

Next, we examined the extent of RF binding directly with anti-cRCP to determine whether the resultant abrogation in its uptake was due to nonspecific protein sequestration. Antibodies were equilibrated with \( ^{3}\text{H}\)-RF at 37°C for 10 min, protein was adsorbed using hydroxyapatite, and protein-associated ligand was then eluted with ethanol. Figure 1C illustrates that RF binds minimally to anti-cRCP and the nonspecific control, BSA, under these conditions. However, binding studies with the known ligand, cRCP, exhibited ~45% of protein-associated ligand, thus substantiating RF recognition for cRCP but not anti-cRCP. This further lends support to the existence and proposed role of a mammalian RCP homologous to the avian form that mediates RF uptake in human placental trophoblasts.

**Recognition and Binding of cRCP and RF Post-Transfection in COS-1 Cells.** BeWo cells express endogenous RCP that contributes to RF absorption when presented in the extracellular media (data not shown). To validate these results, another mammalian cell line, COS-1 (kidney cell line from Green African monkey), was selected for its lack of expression of endogenous RCP. Immunofluorescence microscopy was carried out to ascertain detectable levels of RCP upon transfection with cRCP and cRCP-GFP vectors (Fig. 2A). Functional relevance of these transfections with the secretory RCP was measured using medium binding assays to detect RF-specific interactions in spent culture media. Figure 2B shows that COS-1 cells transfected with cRCP or the GFP-cRCP fusion construct exhibited RF-specific binding as indicated by significant differences between total and nonspecific binding when excess unlabeled RF was added. These results thereby suggest that mammalian cells secrete a RCP-like protein into the extracellular environment that binds and sequesters RF prior to its internalization. Although the cellular accumulation of RF was not enhanced by cRCP in these studies, based on previous results (Fig. 1), we cannot exclude the possibility that this mammalian cRCP-like protein aids in ligand uptake.

**cRCP Traverses the Plasma Membrane to Mediate Internalization of Associated Cargo.** Riboflavin interacts with a specific carrier protein to facilitate its cellular entry,

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CRCP and BSA as positive and negative controls, respectively. Protein anti-cRCP was determined following a 10-min incubation at 37°C using with no inhibitor, preimmune rabbit sera (200 μg/ml), unlabeled RF (1 μM), and BSA (30 μM). B, specificity of anti-cRCP-induced inhibition of RF uptake was assessed by determination of its effect on FA (20 nM) accumulation using unlabeled FA (1 μg/ml) of 125I-cRCP uptake irrespective of species or tissue origin (Fig. 3A). The competitive nature of inhibition indicates that radiolabeled cRCP and cRCP are recognized by identical domains that bind and/or internalize the protein and, thus, can be used as an invaluable tool to define its functional role in RF uptake. It is important to know that the acute exposure of BeWo and LMH/2A cells to the low concentrations of inhibitor (i.e., unlabeled cRCP) resulted in an apparent stimulation of 125I-cRCP but then competed at higher concentrations to interfere with the binding of the labeled substrate. As a result of this trans-stimulation process (exchange of cRCP for 125I-cRCP) in both BeWo and LMH/2A cells, the inhibition values indicated in Fig. 3A may exceed 100% of the control value.

Preliminary studies revealed a 1:1 stoichiometry for RF-cRCP association essential to the vitamin uptake process. Yet, prior experiments in our laboratory suggest that extraneous RF may not be required for the internalization of cRCP (data not shown). To evaluate whether membrane-bound 125I-cRCP is internalized into BeWo and LMH/2A cells, uptake kinetics of 0 to 1.0 μM 125I-cRCP were determined at 37°C. Figure 3B reveals that the uptake process for 125I-cRCP in both cell lines was saturable. Breakdown of the kinetic analyses using eq. 1 exhibits nanomolar affinities for 125I-cRCP in BeWo and LMH/2A cells (Table 1). However, the maximal transport capacity was approximately 3-fold higher in hepatoma cells (Vmax, 75.14 ± 7.6 pmol/mg protein/min) compared with placental trophoblasts (Vmax, 28.56 ± 2.7 pmol/mg protein/min), suggesting differences in endogenous RCP expression and RF-RCP membrane interaction or increased distribution of unidentified RCP cell surface receptors in the liver. Immunoblot analysis of the cell lysate following cRCP uptake revealed a protein band at 37 kDa, which was compared with the pure protein (Fig. 3C). Consequently, the binding of RF to cRCP may drive the entry of this ligand-protein complex into the intracellular domain, where we propose that RF dissociates from the carrier protein to mediate its downstream metabolic effects.

**Fig. 1.** Involvement of a specific carrier and/or receptor in the cellular internalization of riboflavin. A, uptake of [3H]RF (10 nM) was determined in BeWo cells in the presence (20–200 μg/ml) of protein A-purified antibodies raised in rabbits against the cRCP. Controls included incubation with no inhibitor, preimmune rabbit sera (200 μg/ml), unlabeled RF (1 μM), and BSA (30 μM). B, specificity of anti-cRCP-induced inhibition of RF uptake was assessed by determination of its effect on FA (20 nM) accumulation using unlabeled FA (1 μM) and controls as described earlier. C, specific binding of [3H]RF to anti-cRCP was determined following a 10-min incubation at 37°C using cRCP and BSA as positive and negative controls, respectively. Protein but the mechanism that aids ligand uptake upon association remains unknown. Binding and uptake assays were carried out using radiolabeled cRCP in its apo form (without bound RF) to determine whether the protein carrier functions as either a “ligand carrier” that delivers the associated ligand across membrane barriers or via docking onto specific receptors with subsequent release of the bound ligand into the intracellular milieu. Inhibition of 125I-cRCP accumulation using unlabeled cRCP in BeWo cells showed inhibition of the modified protein by its native form, suggesting that the iodination process did not influence recognition by its putative interacting receptor(s). Parallel experiments with control chicken hepatoma (LMH/2A) cells revealed equipotent inhibition (IC50 BeWo = 70.27 ± 30.96 nM; IC50 LMH/2A = 61.08 ± 11.34 nM) of 125I-cRCP uptake irrespective of species or tissue origin (Fig. 3A). The competitive nature of inhibition indicates that radiolabeled cRCP and cRCP are recognized by identical domains that bind and/or internalize the protein and, thus, can be used as an invaluable tool to define its functional role in RF uptake. It is important to know that the acute exposure of BeWo and LMH/2A cells to the low concentrations of inhibitor (i.e., unlabeled cRCP) resulted in an apparent stimulation of 125I-cRCP but then competed at higher concentrations to interfere with the binding of the labeled substrate. As a result of this trans-stimulation process (exchange of cRCP for 125I-cRCP) in both BeWo and LMH/2A cells, the inhibition values indicated in Fig. 3A may exceed 100% of the control value.

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Intracellular Translocation of cRCP via an Endocytic Pathway. Previous studies from our laboratory propose endocytic events in the translocation process of RF (Huang and Swaan, 2000, 2001; Huang et al., 2003). In addition, data from the current study suggest that RF crosses the plasma membrane bound to its carrier protein; hence, to further delineate its cellular trafficking, we evaluated the subcellular distribution profile of this internalized protein.

Cells were coincubated with $^{125}$I-cRCP and $[^3H]$RF, and localization of the dual labels within the cell was assessed via fractionation based on organelle-specific densities. Given the relative densities of the organelles of the endocytic system (i.e., endosomes and lysosomes), only non-nuclear fractions were considered to be relevant. Distribution profiles for RF and cRCP in native LMH/2A (Fig. 4A) cells reveal colocalization of the radiolabeled ligands within fractions that were identified using unique organelle-descriptive markers. Colocalization of $^{125}$I-cRCP and $[^3H]$RF within endosomes of LMH/2A (Fig. 4B) was established via detection of clathrin and Rab5 in fractions 1 to 8 and 10. Moreover, all fractions were positive for LAMP-1 (Fig. 4B), suggesting varying lysosomal sizes within the chicken hepatocytes that are responsible for at least partial degradation of these vesicular contents within the cells. These results definitively show the involvement of endocytic processes in cointernalization of RF associated with its binding protein (RCP) to the intracellular domain.

It is likely that the carrier protein upon dissociation from RF is degraded within the lysosomal compartments (Fig. 4B). We further tested this hypothesis by treating cells with increasing concentrations of a proton ionophore, monensin, known to alter lysosomal accumulation of ligand. This resulted in a 25 to 45% decrease in internalization of prebound $^{125}$I-cRCP; however, the amount of membrane-bound $^{125}$I-cRCP following internalization was significantly increased by 40 to 64% of control (Fig. 5A). This suggests that monensin-treated cells effectively circumvented the degradative process initiated in the lysosomes, resulting in enhanced intracellular accumulation of the complex (cRCP-RF) and decreased internalization via the implicated endocytic cellular absorptive process.

Recycling Events of cRCP. Our findings thus far implicate cRCP in ligand recognition and translocation across the plasma membrane via the hypothesized RF receptor-medi-
ated pathway. As previously indicated, the RF carrier protein is degraded within the lysosomal compartments. However, some protein that escapes lysosomal degradation may recycle back to the surface to follow a retroendocytic mode of transport. To further examine whether the internalized carrier protein undergoes retroendocytosis, recycled amounts of 125I-cRCP were measured over time and as a function of temperature. Figure 5B shows that, at the physiologically relevant temperature of 37°C, 7.12 ± 0.01 pmol of the internalized cRCP recycled back to the outside constituting ~70% of the cellular load. Experimental t1/2 was calculated at 0.97 ± 0.11 min using eq. 2. The amount recycled decreased significantly at the quiescent temperature of 4°C, where only 0.20 ± 0.04 pmol of the cell-loaded cRCP was detected extracellularly after 60 min with a calculated t1/2 of 2.37 ± 0.39 min. At ambient temperatures of 16 to 20°C, previously reported to inhibit lysosomal trafficking (Apodaca et al., 1994; Ellinger et al., 2001), 4.90 ± 0.36 pmol of cRCP was recycled at a t1/2 of 5.18 ± 0.04 min. These data indicate that, although RCP is partially degraded within the cell, some of it is recovered back to the extracellular environment for its continued role in RF internalization.

**Discussion**

RF, commonly referred to as vitamin B2, critically influences the redox status of cells via formation of its phosphorylated derivatives FMN and FAD. Yet, information on the mechanism and process regulators defining the dietary absorption of this essential nutrient remains sparse. Consequently, this lack of understanding provides the impetus to delineate the elements that mediate RF uptake especially in conditions exhibiting enhanced nutritional requirements, such as pregnancy and tumors, which may be further exploited with diagnostic and therapeutic strategies.

Recent studies from our laboratory have indicated a receptor-mediated endocytic mechanism of riboflavin transport in human intestinal and placental cells (Huang and Swaan, 2000, 2001; Huang et al., 2003). In this study, we examine the role of protein chaperones that aid RF endocytosis specific to its recognition and sequestration, followed by cell surface binding and internalization in mammalian cells. The well studied cRCP functions to bind and transport RF. Several laboratories have deduced its presence in mammalian species based on the evolutionary conserved protein domains (Foraker et al., 2003), but a purified human form of the ligand-specific carrier protein presently remains elusive. Here, we demonstrate that human placental trophoblasts depend critically on such a RF-specific carrier protein (RCP) for cellular RF uptake since immunological inhibition with antibodies raised against the chicken RCP results in a total loss of RF accumulation. Indirect immunofluorescence shows that RCP is localized intracellularly in BeWo cells (Fig. 1D), leading us to believe that RCP is secreted by the cell to facilitate RF uptake. Furthermore, this human RCP found in trophoblasts exhibits distinct specificity toward its designated ligand (RF), since cross-reactivity with folate, another water-soluble vitamin, was not established (Fig. 1B).

We next evaluated the mechanism underlying the role of RCP in binding RF and facilitating its internalization process. First, the use of cRCP in a mammalian system was validated by its specific interactions with RF following transfections using cRCP and cRCP-GFP constructs (Fig. 2). We then radiolabeled the apo form of cRCP to serve as a detectable marker and confirmed that these chemical modifications
do not interfere with substrate recognition and binding to its interacting receptor(s) in BeWo cells, as well as in chicken hepatocytes (LMH/2A) that functioned as a species-specific control. Kinetic elucidation of cellular cRCP uptake revealed a saturable process with nanomolar affinities for the integral membrane receptor(s) (Table 1; Fig. 3B), which is in accordance with previous kinetic data of RF from our laboratory (Huang and Swaan, 2001). Based on the aforementioned findings, we propose that RCP serves to sequester its ligand from the extracellular medium and then traverses the membrane barrier with or without the attached cargo.

Differential transport capacities exhibited by the hepatocytes and trophoblasts may be attributed to differences in endogenous RCP expression and membrane interactions with RF or differences in the expression and interaction with unidentified cell surface receptor(s). Although specific RF or RCP receptors have yet to be identified in humans, prior reports allude to cRCP binding with lipoprotein receptors on oocyte membranes (Mac Lachlan et al., 1994). Hence, we sought to examine the role of multiligand-specific apical endocytic receptors belonging to the lipoprotein receptor superfamily, cubulin and megalin (Christensen and Birn, 2002), in RCP-RF internalization. Seemingly, these receptors, although expressed in trophoblasts, do not influence the entry mechanism of RF (data not shown), which suggests that RCP-RF must react with a specific membrane RF receptor to complete the endocytic process. Another possibility that needs to be considered is perhaps the dual role of RCPs that

Fig. 3B. Kinetic characterization of $^{125}$I-cRCP in human placental and chicken liver cells

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Fig. 4. Distribution profiles of $^{125}$I-cRCP and its bound ligand $[^3$H]RF in the postnuclear fractions isolated from chicken hepatocytes. Postnuclear fractions isolated from LMH/2A cells were resolved based on differences in organelle density using a discontinuous sucrose gradient, fractionated, and measured for dual radiolabel accumulation (A) (closed squares, $^{125}$I-cRCP; open circles, $[^3$H]RF). Organelle-enriched fractions 1 to 12 (WC, whole-cell lysate) were identified by immunoblotting (B) using antibodies against endosomal proteins, clathrin and Rab5 GTPase, and the lysosomal marker LAMP-1.

Fig. 5. Effect of monensin and temperature on $^{125}$I-cRCP recycling. A, BeWo cells are pretreated with 2.5 μM monensin followed by coincubation during a 20-min uptake at 37°C. After internalization, membrane-associated and internalized radiolabel was measured by liquid scintillation counting and normalized to protein content. *, monensin-treated cells (solid bars) show significant differences from control (untreated) cells (open bars) at $p \leq 0.01$. B, BeWo cells were dosed with $^{125}$I-cRCP at 37°C, membrane-bound amounts were removed, and recycling was allowed to occur for 1 h at 4°C (squares), 16°C (circles), and 37°C (triangles). Cumulative recycled amounts are depicted as a function of time.
function to sequester the membrane and then dock into the RF receptor.

Visualization of rhodamine-labeled RF by immunofluorescence microscopy coupled with colocalization studies using endosomal and lysosomal markers supports the premise for endocytic mode of ligand entry in human placental cells (Huang et al., 2003). Given that RCP associates with the ligand RF and is then cointernalized, we evaluated the intracellular trafficking profile for the carrier-RF complex. Interestingly, subcellular distribution profiles of RCP within endosomes and lysosomes paralleled those of RF, although intracellular RF concentrations were significantly higher (Fig. 4). This may result from increased rates of RCP degradation and/or recycling to the extracellular environment to acquire additional cargo. This mechanism is analogous to the well-characterized transferrin receptor (TfR), which is endocytosed with its ligand [transferrin (TF)] via clathrin-coated pits. TF remains associated with its receptor (TfR) until it recycles to the plasma membrane at which point dissociation takes place (van Dam et al., 2002). This process distinguishes the TF-TfR complex from other lysosome-targeted proteins.


References


In summary, our experiments demonstrate the presence of a riboflavin carrier protein in human placental trophoblasts that specifically binds its ligand. RF-associated RCP is then recognized by specific membrane binding partners and is taken up into the cells via previously hypothesized endocytic machinery. Internalized RCP traffics within the cell and is ultimately either degraded in the lysosomes or is preserved to recycle back to the surface to scavenge additional cargo. Clearly, the identity of RCP and its receptor need further characterization. Future studies using proteomic tools are aimed at the isolation and identification of RCP and the interacting receptors, which will aid structural and molecular elucidation of the endocytic events.