Mechanisms of provitamin A (carotenoid) and vitamin A (retinol) transport into and out of intestinal Caco-2 cells

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Abstract **The purpose of this study was to compare the mechanisms of intestinal retinol (ROL) and carotenoid transport. When differentiated Caco-2 cells were incubated with ROL for varying times, cellular ROL plateaued within 2 h, whereas retinyl ester (RE) formation increased continuously. ROL and RE efflux into basolateral medium (BM) increased linearly with time, ROL in the nonlipoprotein fraction and REs in chylomicrons (CMs). In contrast to carotenoids, ROL uptake was proportional to ROL concentration (0.5–110 μM). ROL efflux into BM occurred via two processes: *a*) a saturable process at low concentrations (<10 μM) and *b*) a nonsaturable process at higher concentrations. When ROL-loaded cells were maintained on retinoid-free medium, free ROL, but not REs, was secreted into BM. Glyburide significantly reduced ROL efflux but not ROL uptake. Inhibition of ABCA1 protein expression by small interfering RNAs decreased ROL efflux but not carotenoid efflux. Scavenger receptor class B type I (SR-BI) inhibition did not affect ROL transport but decreased carotenoid uptake. The present data suggest that *a*) ROL enters intestinal cells by diffusion, *b*) ROL efflux is partly facilitated, probably by the basolateral transporter ABCA1, and *c*) newly synthesized REs, but not preformed esters, are incorporated into CM and secreted. In contrast to ROL transport, carotenoid uptake is mediated by the apical transporter SR-BI, and carotenoid efflux occurs exclusively via their secretion in CM.—**During, A., and E. H. Harrison. **Mechanisms of provitamin A (carotenoid) and vitamin A (retinol) transport into and out of intestinal Caco-2 cells.** *J. Lipid Res.* **2007.** 48: **2283–2294.**

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Vitamin A [or retinol (ROL)] is an essential fat-soluble nutrient required by humans for vision, cellular differentiation, development and growth, reproduction, and immunity (1). The de novo synthesis of vitamin A is limited to plants and microorganisms. Thus, humans must obtain vitamin A from the diet, either as preformed ROL or as provitamin A carotenoid precursors. Both deficiency and excess of vitamin A are known to cause pathologies. Vitamin A deficiency is usually a result of malnutrition but can also be attributable to abnormalities in the intestinal absorption of ROL or provitamin A carotenoids. Vitamin A deficiency especially affects children (100 to 140 million) in >100 countries (2). Vitamin A excess, although not as common as deficiency, is a result of excessive supplementation. Therefore, better knowledge about the mechanisms of absorption of vitamin A is important in determining the appropriate amounts of vitamin A required by humans.

Vitamin A is present in the diet as retinyl esters (REs) found in foods of animal origin and as provitamin A carotenoids (mainly β-carotene, α-carotene, and β-cryptoxanthin) found in plant-derived products. Intestinal absorption of vitamin A from these dietary compounds requires several enzymatic steps. The hydrolysis of dietary REs in the intestinal lumen is catalyzed by enzymes such as the pancreatic triglyceride (TG) lipase and the intestinal brush border phospholipase B to yield unesterified ROL, which is the form absorbed into the intestinal mucosal cell. Some of the newly absorbed ROL undergoes reesterification with long-chain, mainly saturated, fatty acids, which is catalyzed by lecithin:retinol acyltransferase. The oxidative cleavage of carotenoids in intestinal cells to form retinal is catalyzed by the β-carotene 15,15′-oxygenase, followed by the reduction of retinal to ROL catalyzed by the retinal reductase (for reviews, see Refs. 3–5). Thus, these different enzymes mediate distinct metabolic pathways for dietary REs and provitamin A carotenoids that converge to form both ROL and REs in intestinal cells. The formed REs and noncleaved carotenoids are then incorporated into chylomicrons (CMs) before their secretion into the lymph circulation (6) and unesterified ROL can directly enter the portal circulation.

Abbreviations: ABCA1, ATP-binding cassette, sub-family A, member 1; AM, apical medium; apoB, apolipoprotein B; BM, basolateral medium; CM, chylomicron; LP2000, Lipofectamine™ 2000; NPC1L1, Niemann-Pick disease type C1 gene-like 1; OA, oleic acid; RE, retinyl ester; ROL, retinol; siRNA or RNAi, small interfering RNA; SR-BI, scavenger receptor class B type I; TC, taurocholate; TG, triglyceride.

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Recent discoveries of several protein transporters involved in the intestinal absorption of cholesterol have completely modified the previously held view that lipids are transported through the intestinal cells by passive diffusion. A facilitated process has been clearly suggested for cellular ROL uptake, although the protein transporter involved has not been identified. For instance, early studies using rat intestinal segments suggested that free ROL was taken up by a saturable, carrier-mediated process at physiological concentrations (<150 nM) and by a diffusion process at pharmacological concentrations (450–2,700 nM) (7, 8). In the human intestinal cell line Caco-2, the observed biphasic production of REs by cells was also attributed to two processes: via a saturable, carrier-mediated process at lower ROL concentrations (0.5–8 μM) and a nonsaturable, diffusion-dependent process at higher concentrations (8–30 μM) (9). Similar conclusions were drawn from studies of intact rat intestinal preparations (10, 11).

Recent reports have indicated that the intestinal absorption of carotenoids is a facilitated process, but the exact number and identity of the transporters involved is still under study. β-Carotene transport into Caco-2 cells was characterized as a saturable, concentration-dependent process with isomer specificity (12), and the participation of the scavenger receptor class B type I (SR-BI) in the intestinal transport of carotenoids was later reported in Caco-2/TC-7 cells (13) and in wild-type versus SR-BI knockout mice (14). Our own recent data suggested that carotenoid trafficking in intestinal cells may also involve more than one ezetimibe-sensitive transporter (15), such as SR-BI, Niemann-Pick disease type C1 gene-like 1 (NPC1L1), and ABCA1.

In the work reported here, we sought to further define the mechanisms of the intestinal absorption of dietary carotenoids and retinoids with a focus on defining potential membrane transport mechanisms.

MATERIALS AND METHODS

Chemicals

All-*trans*-ROL, all-*trans*-β-carotene (type IV, >95% purity), oleic acid (OA), taurocholate (TC), glycerol, Tween 40, glyburide (or glybenclamide), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All-*trans*-ROL was purified (>98%) by HPLC on a TSK Gel Silica 60 column (4.6 × 250 mm) using hexane-ethyl acetate (80:20, v/v) as the mobile phase. Lutein and β-cryptoxanthin (>99.9% pure compounds by TLC) were from Indofine Chemical Co., Inc. (Hillsborough, NJ). Rabbit polyclonal antibodies against human SR-BI and ABCA1 were from Novus Biologicals (Littletown, CO), and rabbit polyclonal antibodies against human NPC1L1 were from Cayman Chemical (Ann Arbor, MI). [1,2,3-^{3}H]glycerol (53.5 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA).

Cell culture

Caco-2 cells (passages 30–50) were obtained from the American Type Culture Collection (Rockville, MD) and grown as described previously (12). For experiments, cells were plated on six-well plates or Transwells (24 mm diameter, 3 μm pore size; Corning Costar Corp., Cambridge, MA) in the presence of complete medium [DMEM plus 20% heat-inactivated FBS, 1% nonessential amino acids, and 1% antibiotics (Gibco Life Technologies, Inc.)] for 3 weeks.

Before the transport experiments on Transwells, the integrity of the cell monolayer was tested either by determining the diffusion of phenol red from the apical side to the basolateral side or by measuring the resistance of the cell monolayer with a Millicell® Electrical Resistance System (Millipore). At zero time, the apical side received 2 ml of serum-free medium (DMEM with 1% nonessential amino acids) supplemented with 0.5 mM TC, 1.6 mM OA, and 45 μM glycerol or [1,2,3-^{3}H]glycerol plus other compounds such as ROL, carotenoids, and glyburide. The basolateral side received 2 ml of serum-free medium. After incubation at 37°C, media were collected from each side of the membrane. Apical medium (AM) was stored at −20°C until analyses, and basolateral medium (BM) was subjected to differential density gradient centrifugation to isolate lipoprotein fractions. The cell monolayer was washed three times with 2 ml of Hanks' balanced salt solution, and total lipids were extracted. Before RNAi transfection experiments, cells were washed twice with saline Hanks' solution before the addition of Opti-MEM® I reduced-serum medium (Gibco).

Delivery of ROL and carotenoids to cells

ROL, β-carotene, lutein, or β-cryptoxanthin were delivered to cells as described previously (16) using 0.1% Tween 40 (final concentration). Final concentrations of ROL and carotenoids applied to the AM are given below.

Addition of glyburide to the cell culture medium

Glyburide at a final concentration of 0.2 or 1.0 mM was solubilized in serum-free medium supplemented with TC/OA at 10:32 mM, followed by an agitation for 1 h at 25–30°C. The resulting solution was sterilized by passage through a 0.22 μm pore size filter.

Lipoprotein fractionation

Large CM, small CM, and VLDL were isolated from the BM by sequential density gradient ultracentrifugation as described previously (17). Briefly, 3.5 ml of media was well mixed with 0.57 g of KBr (density of 1.1 g/ml) and then overlaid with 3 ml each of 1.063 and 1.019 g/ml, and 2 ml of 1.006 g/ml, density solutions using an Auto Densi-Flow (Labconco Corp.). The sample was subjected to three successive centrifugations at 15°C (SW41 rotor; Beckman Instruments, Inc.): *a*) 270,000 *g* (or 40,000 rpm) for 33 min; *b*) 270,000 *g* for 3 h 28 min; and *c*) 270,000 *g* for 17 h (17). After each centrifugation, 1 ml was collected from the top corresponding to large CM (Svedberg flotation > 400), small CM (Svedberg flotation = 60–400), and VLDL (d < 1.006 g/ml, Svedberg flotation = 20–60) fractions, respectively, and replenished with 1 ml of 1.006 g/ml density solution before the next centrifugation.

Lipid extraction

Total lipids (including retinoids and carotenoids) were extracted from cell monolayers as described previously (12) according to Barua and Olson (18). The resultant lipid extract in 1 ml of methanol-dichloromethane (84:16, v/v) was kept at −20°C until TG, retinoid, and carotenoid analyses. These analyses were usually performed within 24 h after extraction. Lipids were extracted from cell culture media (before and after treatment) and the different lipoprotein fractions using the method of Folch, Lees, and Sloane-Stanley (19). Recoveries of the

compound of interest (TG, carotenoids, or retinoids) were followed in each experiment by comparing the dose applied at the apical side of cells (at zero time) and the amounts of compound plus its metabolites recovered in cells and apical and basolateral media after incubation. Total recoveries generally exceeded 85%.

Lipid analysis

[1,2,3-^{3}H]glycerol was added to the AM of cells to label and quantify the newly formed TG and phospholipid pools. Total lipids extracted from cells, media, and lipoprotein fractions were separated by TLC (Silica Gel 60, 0.5 mm thick; Merck) as described previously (12). Lipid bands corresponding to TG and phospholipids were first visualized with iodine vapor, scraped from the plate, dissolved in 5 ml of scintillation cocktail liquid, and analyzed for ^{3}H.

Retinoid extraction and analysis by HPLC

Extraction of retinoids from liquid samples was carried out by a procedure described previously (20). To 1 volume of sample was added 1 volume of ethanol, 1 volume of ethyl acetate, and 1 volume of hexane. The mixture was vigorously mixed and centrifuged for 1 min at 10,000 rpm. The supernatant was kept on ice, and the lower phase was extracted two more times with hexane. The three hexane phases were pooled, the solvent was evaporated, and the residue was dissolved in 300 μl of acetonitrile-dichloromethane (7:3, v/v) and applied to the HPLC system. Extraction of retinoids from cell monolayers was realized as indicated above (see Lipid extraction).

Retinoids were analyzed using a Waters (Milford, MA) HPLC system equipped with a model 717-plus autosampler, a model 996 photo diode array detector, and a Millenium^{32} chromatography manager (Waters T System). Retinoids were eluted on a TSK Gel ODS 120-A C18 reverse phase column, 4.6 × 250 mm (TosoHaas, Montgomeryville, PA), using the following program: 0–6 min, isocratic 100% methanol at 0.9 ml/min; 6–12 min, gradient to methanol-dichloromethane (84:16, v/v) at 1.3 ml/min; 13–30 min, isocratic methanol-dichloromethane (84:16, v/v) at 1.0 ml/min; and finally, 30–35 min, gradient to 100% methanol at 1.0 ml/min. Retention times of ROL, retinyl palmitoleate, linoleate, oleate, palmitate, and stearate were 5.6, 16.5, 17.3, 21.0, 23.8, and 27.2 min, respectively. Each retinoid was quantified from its peak area by comparison with a standard reference curve established with different amounts of the respective standard retinoids (from 0.5 to 500 pmol) in methanol-dichloromethane (84:16, v/v) at 325 nm.

RE syntheses

RE standards were synthesized according to the acyl chloride procedure of Huang and Goodman (21) as adapted by Ross (22) using ~0.12 mmol of ROL and ~0.14 mmol of acyl chloride for each ester synthesis. Each RE formed (retinyl palmitoleate, linoleate, oleate, palmitate, and stearate) was extracted in hexane and passed through an alumina column (grade III, 6% water) using 30 ml of hexane as the elution solvent. The resultant RE solution was analyzed for its concentration by spectrophotometry and for its purity by HPLC (see above for HPLC conditions). The purity of each RE standard was >95%.

Carotenoid extraction and analysis by HPLC

Carotenoids were extracted from cells and media as described previously (18). Carotenoids were analyzed using the HPLC system and column that were used for retinoid analysis (see above) but in presence of the following mobile phase: methanol-dichloromethane (84:16, v/v) at 1 ml/min, and monitored at 450nm.

Small interfering RNA inhibition of SR-BI, NPC1L1, and ABCA1

Gene expression of SR-BI, NPC1L1, and ABCA1 in Caco-2 cells was blocked using small interfering RNAs (siRNA or RNAi). For each protein targeted, three double-stranded Stealth™ RNAs were used (see sequences in **Table 1**) that were designed by the BLOCK-iT™ RNAi Designer (Invitrogen Corp., Life Technologies, Carlsbad, CA). Stealth™ scrambled siRNAs, Low GC Duplex (36% GC), and Medium GC Duplex (48% GC) (Invitrogen Corp.) were used as negative controls. RNAi transfection into Caco-2 cells was done using Lipofectamine™ 2000 (LP2000) (Invitrogen Corp.). RNAi-LP2000 complexes were formed by adding 5 μl of LP2000 and 250 pmol of RNAi in Opti-MEM® I reduced-serum medium. RNAi-LP2000 complexes were added to cells, followed by incubation at 37°C. At 72 or 96 h after transfection, cells were incubated with ROL or individual carotenoids (2 μM) for 1 or 4 h, when cellular uptake (for SR-BI and NPC1L1

TABLE 1. Sequences of RNAi used to target the human lipid transporters SR-BI, NPC1L1, and ABCA1

Gene Targeted (Human)	*Accession Number*	*Name of RNAi*	*Sequences of RNAi*
SR-BI	NM_005505	RNAi_667	Sense, 5′-CCC UUC AAG GAC AAG UUC GGA UUA U Antisense, 5′-AUA AUC CGA ACU UGU CCU UGA AGG G
		RNAi_1461	Sense, 5′-CCG GAG CCA AGA GAA AUG CUA UUU A Antisense, 5′-UAA AUA GCA UUU CUC UUG GCU CCG G
		RNAi_1850	Sense, 5′-UCA ACA AGC ACU GUU CUG GAA CCU U Antisense, 5′-AAG GUU CCA GAA CAG UGC UUG UUG A
NPC1L1	NM_013389	RNAi_1831	Sense, 5′-GGG AGG AGG CCU UCU UAG AGG AAA U Antisense, 5′-AUU UCC UCU AAG AAG GCC UCC UCC C
		RNAi_3832	Sense, 5′-CCC AGC UCA UUC AGA UCU UCU UCU U Antisense, 5′-AAG AAG AAG AUC UGA AUG AGC UGG G
		RNAi_4401	Sense, 5′-GCA GGA ACA GAC ACA CUC CAU GUU U Antisense, 5′-AAA CAU GGA GUG UGU CUG UUC CUG C
ABCA1	NM_005502	RNAi_4943	Sense, 5′-GCA CUU CCU CCG AGU CAA GAA GUUA Antisense, 5′-UAA CUU CUU GAC UCG GAG GAA GUGC
		RNAi_6086	Sense, 5′-CGG AAG CCU GCU GUU GAC AGG AUU U Antisense, 5′-AAA UCC UGU CAA CAG CAG GCU UCC G
		RNAi_6838	Sense, 5′- GAC CAA AGU GAU GAU GAC CAC UUA A Antisense, 5′-UUA AGU GGU CAU CAU CAC UUU GGU C

NPC1L1, Niemann-Pick disease type C1 gene-like 1; RNAi, small interfering RNA; SR-BI, scavenger receptor class B type I.

inhibition) and secretion into the BM (for ABCA1 inhibition) were studied, respectively.

Western blot analysis of SR-BI, NPC1L1, and ABCA1

Cells were plated in 25 cm^2 flasks and then transfected with the corresponding RNAi. At 72 to 96 h after transfection, medium was removed, cells were washed three times with a saline solution, and proteins were extracted using a total protein extraction kit (Chemicon International, Inc.). Protein concentration of samples was then determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories), and 25–50 μg of proteins was used for Western blot analysis. Western blot analyses were done according to the NuPAGE® technical guide (Invitrogen Corp.). Proteins were separated by SDS-PAGE under reducing conditions using a 4–12% NuPAGE® Novex Bis-Tris gel (for SR-BI) or a 3–8% NuPAGE® Novex Tris-Acetate gel (for NPC1L1 and ABCA1) with NuPAGE MOPS or Tris-Acetate SDS running buffer. After electrophoresis, proteins were transferred onto a 0.45 μm nitrocellulose membrane. Blotted membranes were then incubated with the anti-human IgG primary antibodies against SR-BI at 1:500, ABCA1 at 1:250, or NPC1L1 at 1:250, and immunodetection was performed using an anti-rabbit IgG secondary antibody according to the WesternBreeze Chromogenic Kit (Invitrogen Corp.).

Statistical analysis

All data are expressed as means ± SD. Statistical analysis of results was assessed by one-way ANOVA coupled with the Fisher's test. Relationships between two variables were examined by simple or logarithmic regression analyses. The choice of the regression (simple vs. logarithmic or exponential) was determined by the squared value of the regression coefficient (R^2); the regression given the highest R^2 value was chosen. All statistical analyses were performed using Statview version 5.0 (SAS Institute, Cary, NC). A value of $P \leq 0.05$ was considered significant.

RESULTS

Kinetics of ROL uptake, esterification, and secretion into lipoproteins as a function of incubation time

Differentiated Caco-2 cell monolayers were incubated with 3 μM ROL for 1–24 h. Cellular ROL plateaued within 2 h, whereas RE formation increased linearly with time of incubation up to 24 h ($R^2 = 0.998$, $P < 0.0001$, n = 9 time points) (**Fig. 1A**). An esterification rate of ROL was estimated as 70 pmol RE formed per hour per 2×10^6 Caco-2 cells when incubated with 3 μM ROL. Under standard conditions (using OA and Tween 40), four main REs were identified: retinyl linoleate (1%), retinyl oleate (70%), retinyl palmitate (25%), and retinyl stearate (3%) (percentage of total REs), and these proportions were unchanged with incubation time (data not shown). The amounts of ROL and REs secreted into the BM increased linearly for up to 20 h and then appeared to plateau (Fig. 1B). An initial lag phase of 2 h was observed for RE secretion but not for ROL secretion. Only retinyl oleate (57%) and retinyl palmitate (43%) were detected in BM. Similarly, the main retinoids secreted in human CM after the intake of fat-rich meals were retinyl palmitate and oleate (63–79% of total REs) (23). In this study,

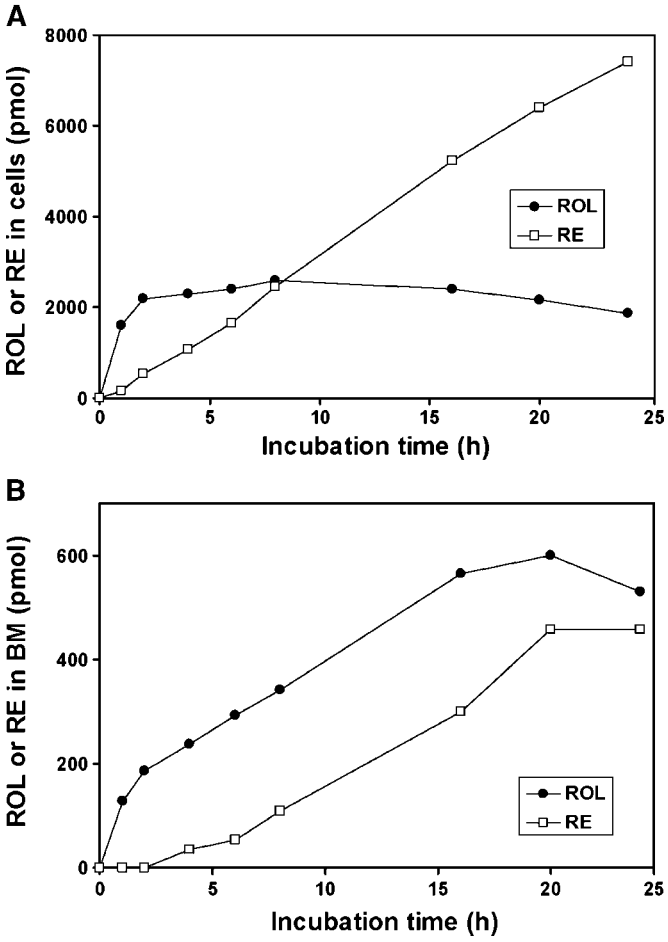

Fig. 1. Effects of incubation time on the cellular uptake and intracellular esterification of retinol (ROL) (A) and on the secretion into the basolateral medium (BM) of ROL and retinyl ester (RE) (B). At zero time, 3 μM ROL in the presence of oleic acid-taurocholate (OA/TC) at 0.5:1.6 mM was added to apical medium (AM) of differentiated Caco-2 cells. At the indicated times, cells and BM were analyzed for their content of ROL and RE. Data are means of duplicate or triplicate assays obtained from one experiment (variation between values was <5% of the mean).

the relatively high level of retinyl oleate secreted is probably related to the OA supplementation. As expected, the majority of REs (70% of total RE secreted) was associated with the TG-rich lipoprotein fractions, CM and VLDL. REs increased linearly in these two lipoprotein fractions with time up to 20 h (**Fig. 2A**). In contrast, the majority of ROL (70% of total free ROL secreted) was recovered in the "rest" fraction and increased up to 16 h (Fig. 2B).

Kinetics of ROL uptake, esterification, and secretion: effect of apical ROL concentration

When differentiated Caco-2 cells were incubated for 16 h with ROL (0.2–110 μM), cellular ROL increased linearly with the initial concentration of ROL. Best-fitting curves were obtained by linear regression analysis for the wider concentration range of 0.2–110 μM ($R^2 = 0.991$, n = 43 points) as for the smaller concentration range of

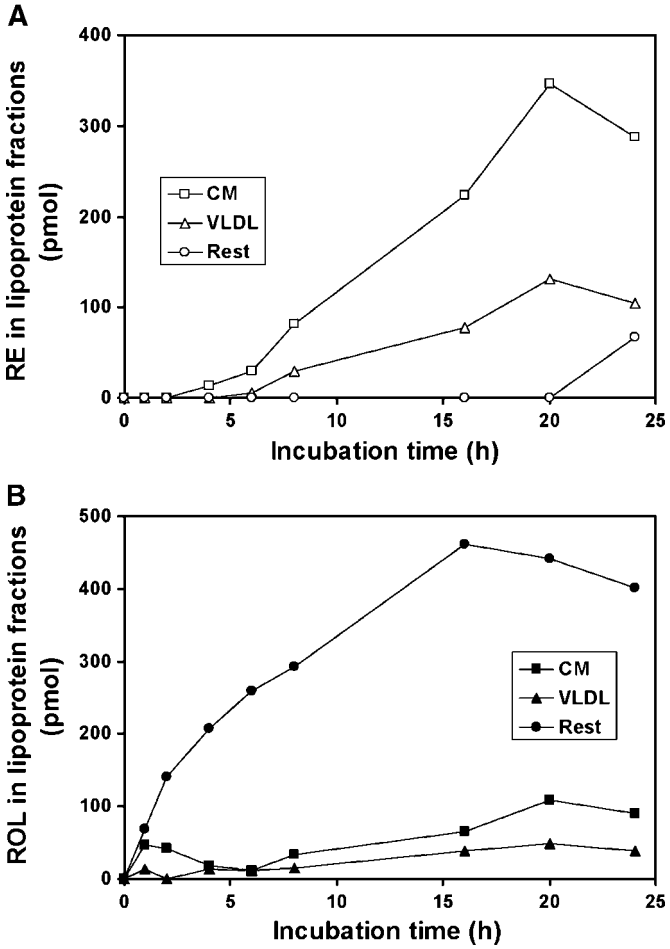

Fig. 2. Effects of incubation time on the levels of RE (A) and ROL (B) in different lipoprotein fractions [chylomicrons (CMs; squares), VLDL (triangles), and nonlipoprotein fraction (Rest; circles)] in BM. At zero time, cells were incubated with 3 μM ROL in medium with OA/TC at 0.5:1.6 mM for varying times. At the indicated times, the BM was subjected to density gradient fractionation and the ROL and RE contents of the lipoprotein fractions were determined. Data are means of duplicate or triplicate assays obtained from one experiment (variation between values was <5% of the mean).

0.2–10 μM (R^2 = 0.800, n = 24 points) (**Fig. 3A**). ROL in the BM increased according to a power function of the initial ROL concentration (0.2–110 μM) (R^2 = 0.959, n = 43 points, $P < 0.0001$) (Fig. 3B), suggesting that ROL transport across the basolateral membrane of Caco-2 cells is dependent on two processes: a diffusion process and facilitated transport. Indeed, according to the model of Neame and Richards (24), once the transporter is saturated the substrate transfer will be attributed almost entirely to diffusion upon further increases in concentration. In this study, at low (i.e., physiological) concentrations, ROL secretion into the BM was a logarithmic function of ROL concentration [y = 372.Ln(x) + 365; R^2 = 0.853, n = 24 points, $P < 0.0001$], with a plateau reached at 10 μM ROL. This suggests a saturable process with an apparent K_m of 3 μM and a V_{max} of 90 pmol ROL secreted per hour per 2 × 10^6 Caco-2 cells. Similar apparent K_m values for ROL uptake were observed previously in Caco-2 cells (K_m = 3 μM) (9) and in everted gut sacs from adult rat jejunum (K_m = 8 μM) (10).

At pharmacological concentrations (11–110 μM), ROL secretion was a linear function of ROL concentration (y = 58.x + 1,270; R^2 = 0.919, n = 19 points, $P < 0.0001$). This suggests a passive diffusion process with a diffusion rate of 2 mmol ROL secreted/h/mol ROL added to the apical side of 2 × 10^6 Caco-2 cells. RE formation in cells was proportional to the initial ROL concentration for the range of 0.2–110 μM (y = 675.x + 179; R^2 = 0.983) as well as for the physiological range of 0.2–10 μM (y = 684.x − 59; R^2 = 0.876) (data not shown). In addition, the secretion of total REs and individual REs into BM was a linear function of apical ROL concentration (data not shown). Thus, these data suggest that ROL flux through intestinal cells is at least partly a facilitated transport that occurs preferentially at the basolateral side of the cells. This conclusion is supported by the observation that ROL flux was significantly lower (1.9 ± 1.0%) from the basolateral side to the apical side than in the opposite direction (9.4 ± 2.9%; n = 4).

Distribution of ROL and REs in cells, BM, and the different lipoprotein fractions

After 16 h of incubation with concentrations of <10 μM, total ROL (ROL + RE) in cells was greater (60%) than that in AM (24%) and BM (16%) (**Table 2**). In cells, 50% of ROL was free and 50% was esterified. The presence of mRNA for lecithin:retinol acyl transferase (the enzyme catalyzing the ROL esterification) in Caco-2 cells was confirmed by real-time PCR analysis. Its expression was half that found in a human small intestine sample (15). In BM, 80% of ROL was free and 20% was present as RE. Approximately 70% of total free ROL secreted was associated with the nonlipoprotein fraction, whereas almost 80% of total RE secreted was associated with TG-rich lipoprotein fractions (CM + VLDL) (Table 2). Thus, of the 16% of total ROL passing through Caco-2 cells, half was present as free ROL in the nonlipoprotein fraction, indicating that the portal circulation could be a major route for ROL delivery to the liver in vivo.

Lack of secretion of preformed cellular REs

Cells were first incubated with ROL for 16 h to accumulate cellular ROL and REs, followed by incubation with ROL-free medium (containing fatty acids and thus mimicking the "fed" state). Medium was changed every 24 h for up to 5 days to study the release of intracellular ROL and REs. Data are shown in **Fig. 4**. After incubation with 3 μM ROL for 16 h, cells accumulated approximately two times more REs than ROL, as also shown in Fig. 1. These data constituted the baseline of the experiment. Thereafter, when maintaining cells in a ROL-free medium, ROL in cells decreased with time in an inverse logarithmic relation [y = −593.Ln(x) + 2,938; R^2 = 0.887] and ROL in BM increased with time in a positive logarithmic relation [y = 563.Ln(x) − 970; R^2 = 979], indicating clearly that cellular ROL was progressively released from

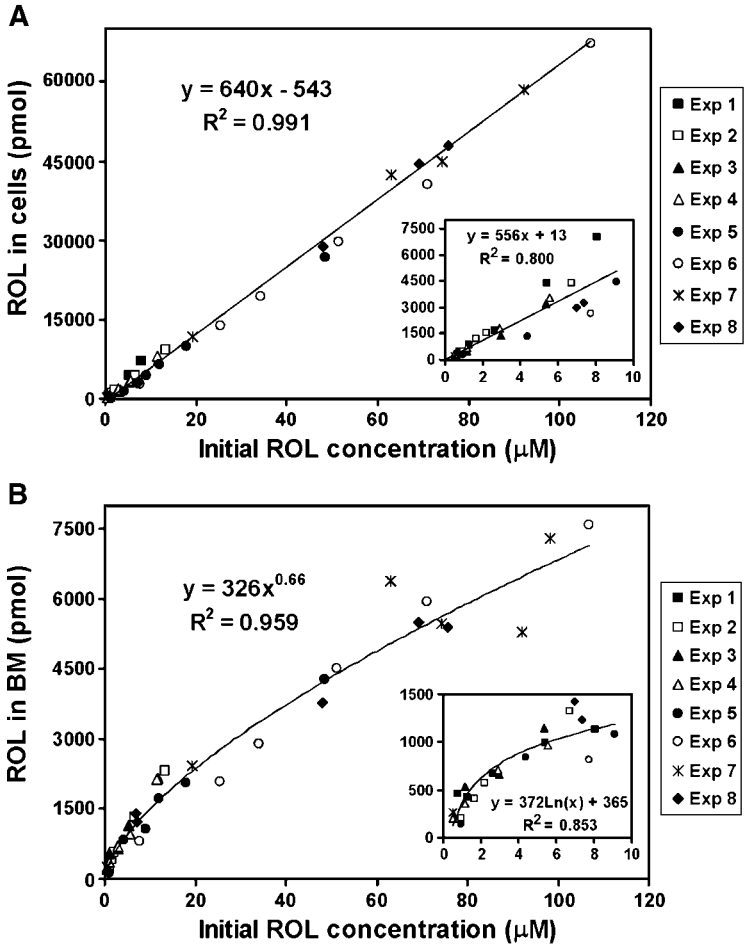

Fig. 3. Effects of the initial concentration of ROL (0.2–110 μM) added to the AM of Caco-2 cells on the cellular uptake of ROL (A) and its secretion into the BM (B). Insets represent data for the ROL concentration range of 0.2–10 μM. At zero time, cells were incubated with varying ROL concentrations in the presence of OA/TC at 0.5:1.6 mM. After 16 h of incubation, cells and BM were analyzed for their ROL content. Data are means of duplicate or triplicate assays obtained from eight independent experiments (variation between values was <5% of the mean).

the cells and secreted into BM after each "feeding" period. Amounts of REs in cells and in BM were unchanged during the 5 days of the washout experiment, suggesting that, in contrast to ROL, REs were not released from cells after a feeding period and thus remained in the cell.

Effect of glyburide on ROL transport in Caco-2 cells

Glyburide (also called glybenclamide) (**Fig. 5**) was chosen for its activity as an inhibitor of ABC proteins, in particular ABCA1. Inhibition of ABCA1 by glyburide has been reported to occur in the concentration range of 0.1–1 mM in cultured cells (25–27). In this experiment, the effect of glyburide on ROL transport by differentiated Caco-2 cells was tested at 0.2 and 1 mM. Although glyburide had no significant effect on cellular ROL content (Fig. 5A), ROL level in BM was decreased significantly in a dose-dependent manner (by 36% and 62%, respectively, with 0.2 and 1 mM glyburide) (Fig. 5B). These data

TABLE 2. Distribution of ROL and RE in AM, cells, and BM for physiological ROL concentrations (0.2–10 μM) and in the different lipoprotein fractions for a wider ROL concentration range (0.5–110 μM) after 16 h of incubation

Distribution in the Different Compartments[a]	AM	Cells	BM
Total ROL (%)	23.5 ± 10.7	60.6 ± 13.2	15.9 ± 6.4
ROL (% of total of ROL + RE)	100	46.8	80.0
RE (% of total of ROL + RE)	0	53.2	20.0
Distribution in the Lipoprotein Fractions from Total Secreted[b]	Chylomicron (Sf > 60)	VLDL (d < 1.006 g/ml, Sf = 20-60)	Rest (d > 1.006 g/ml)
% of total ROL	24.9 ± 8.2	7.1 ± 3.2	68.0 ± 9.7
% of total RE	65.2 ± 14.0	15.8 ± 7.4	19.0 ± 8.3

AM, apical medium; BM, basolateral medium; RE, retinyl ester; ROL, retinol; Sf, Svedberg flotation.

[a] Percentage of total ROL (ROL + RE) in cells and BM was determined from total ROL recovered after incubation (means ± SD, n = 24 points).

[b] Percentage of free ROL and RE in the different lipoprotein fractions was calculated from ROL and RE recovered after ultracentrifugations (means ± SD, n = 18 points).

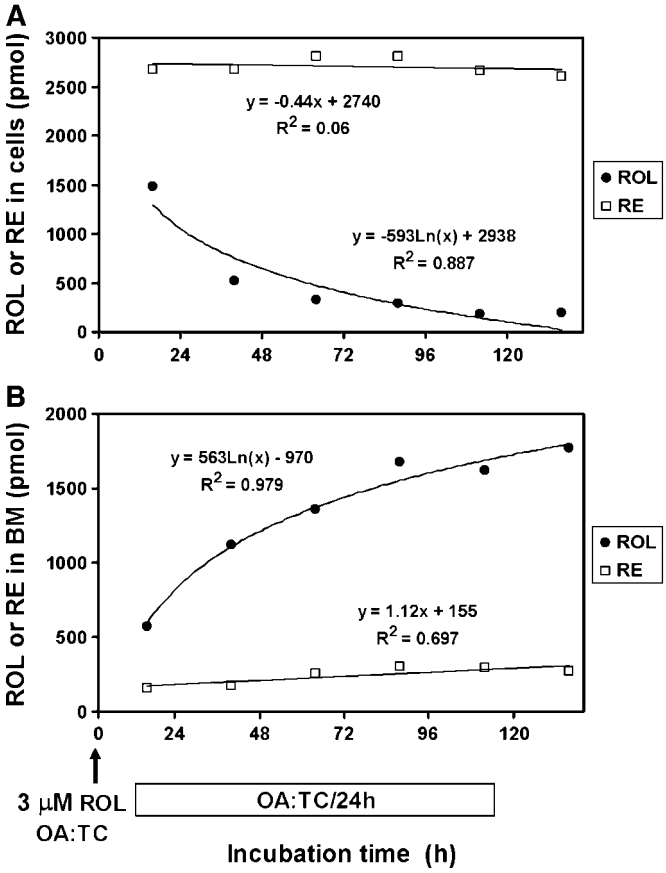

Fig. 4. Fates of ROL and REs in Caco-2 cells (A) and in the BM (B) after their cellular accumulation. At zero time, cells were incubated with 3 μM ROL in the presence of OA/TC at 0.5:1.6 mM for 16 h to accumulate cellular retinoids. After this time, the AM was changed every 24 h and replaced by a fresh, ROL-free medium containing TC/OA. Cells and BM were analyzed for ROL and RE at different times (16, 40, 64, 88, 112, and 136 h). Data are means of duplicate or triplicate assays obtained from one experiment (variation between values was <5% of the mean).

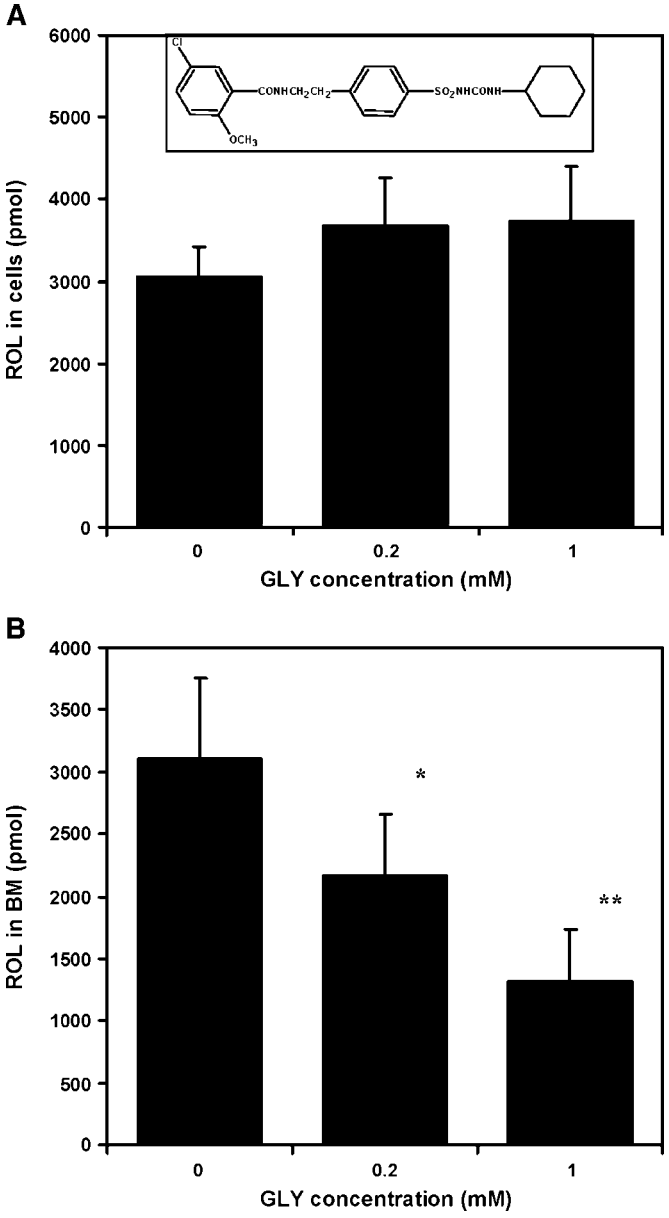

Fig. 5. Effects of glyburide (GLY) on ROL uptake by Caco-2 cells (A) and on ROL secretion (B). Differentiated cells were incubated with 5 μM ROL in the presence or absence of glyburide at concentrations of 0.2 and 1 mM for 16 h. After incubation, ROL contents in cells and BM were determined. Data are means ± SD of three independent experiments. Data were statistically different, with * $P < 0.05$ and ** $P < 0.005$ compared with the control (0 mM glyburide).

indicate that glyburide affects ROL transport at the basolateral membrane, but not at the apical membrane, and support the likelihood that the transport of ROL across intestinal cells is partly facilitated.

Effect of SR-BI, NPC1L1, and ABCA1 gene silencing on ROL and carotenoid transport

For each gene targeted (SR-BI, NPC1L1, and ABCA1), three siRNAs of 25 nucleotides (see sequences in Table 1) were tested to ensure that at least one of the three sequences exhibited good knockdown properties. Caco-2 cells were thus transfected with a siRNA, and the expression of the targeted protein was analyzed by Western blotting (**Figs. 6A**, **7A**, **8A**). The nine siRNAs tested exhibited knockdown properties, as seen by the expression levels of the three proteins, SR-BI, NPC1L1, and ABCA1, compared with their levels in control cells not treated with siRNA. However, in each case, knockdown of the three proteins was incomplete: NPC1L1 (~70% knockdown) > SR-BI (~40%) > ABCA1 (~30%), probably as a result of the relatively low efficiency of the cationic lipid (LP2000)-mediated delivery of siRNAs to Caco-2 cells. The transfection efficiency of a RNAi-oligonucleotide marker was slightly higher in cells grown on plates (~40%) than in cells grown on Transwells (~30%), but in both cases it was relatively low.

Knockdown of SR-BI did not affect ROL uptake by Caco-2 cells but resulted in the decreased uptake of the three carotenoids compared with the negative controls ($P < 0.05$) (Fig. 6B). Of the three siRNAs used for SR-BI knock-

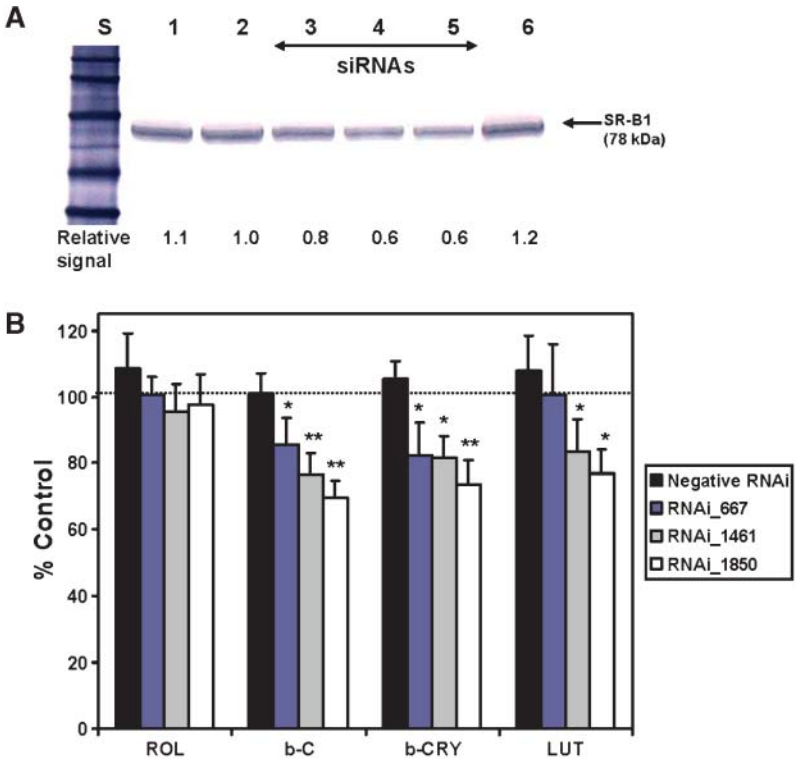

Fig. 6. Effects of the small interfering RNA (siRNA or RNAi) inhibition of scavenger receptor class B type I (SR-BI) expression on the cellular uptake of ROL, β-carotene (b-C), β-cryptoxanthin (b-CRY), or lutein (LUT) in Caco-2 cells. A: Immunoblots of SR-BI expression (using 25 μg total protein/well) in cells treated under the following conditions: lane 1, scrambled RNAi; lane 2, Lipofectamine™ 2000 (LP2000) only; lane 3, RNAi_667; lane 4, RNAi_1461; lane 5, RNAi_1850; lane 6, no treatment. Lane S represents protein standards (MagicMark XP standard from 60 to 220 kDa). B: Cellular uptake of ROL and carotenoids (expressed as the percentage of control cells treated with LP2000 only) after incubation of cells with ROL or a carotenoid at 2 μM for 1 h at 72 h post transfection with a RNAi against SR-BI. Data are means ± SD of three to five independent experiments for each compound tested. * $P < 0.05$, ** $P < 0.0001$ compared with the negative control.

down, RNAi_1850 was the most efficient; indeed, cellular uptake was reduced by 30% for β-carotene, 26% for β-cryptoxanthin, and 23% for lutein. Note that the amount of uptake inhibition was quantitatively similar to the extent of knockdown. RNAi_667 was the least efficient of siRNAs against SR-BI, reducing or not the cellular uptake of the three carotenoids (0–18%) and decreasing the level of expression of SR-BI to a lesser extent than the two others siRNAs (RNAi_1461 and RNAi_1850) (Fig. 6A).

Knockdown of NPC1L1 did not have any effect on the cellular uptake of either ROL or the three carotenoids tested (Fig. 7B), despite the relatively high extent of

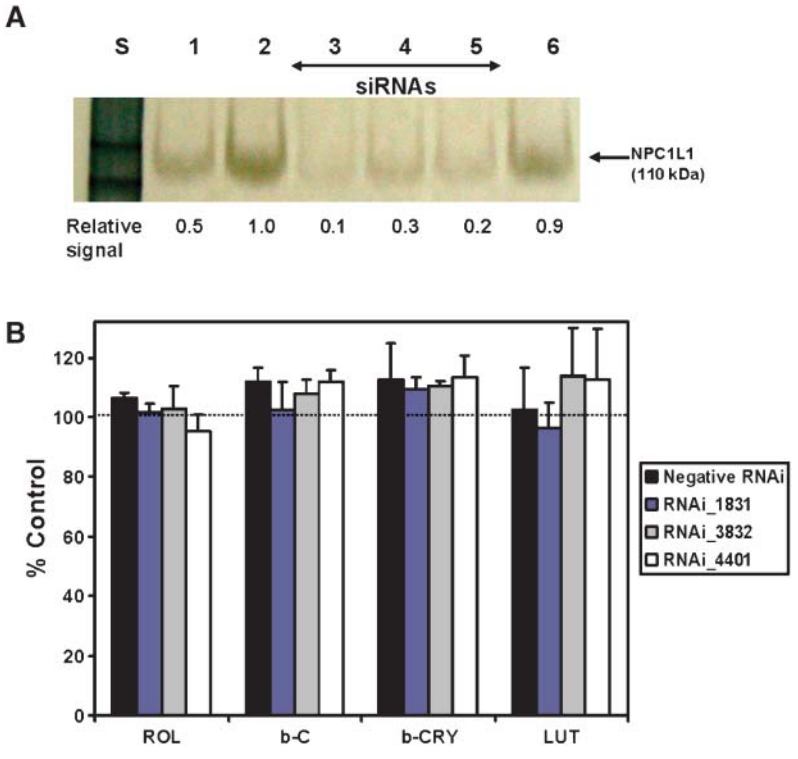

Fig. 7. Effects of the siRNA inhibition of Niemann-Pick disease type C1 gene-like 1 (NPC1L1) expression on the cellular uptake of ROL, β-carotene (b-C), β-cryptoxanthin (b-CRY), or lutein (LUT) in Caco-2 cells. A: Immunoblots of NPC1L1 expression (using 25 μg total protein/well) in cells treated under the following conditions: lane 1, scrambled RNAi; lane 2, LP2000 only; lane 3, RNAi_1831; lane 4, RNAi_3832; lane 5, RNAi_4401; lane 6, no treatment. Lane S represents protein standards (HiMark™ prestained standard from 55 to 460 kDa). B: Cellular uptake of ROL and carotenoids (expressed as the percentage of control cells treated with LP2000 only) after incubation of cells with ROL or a carotenoid at 2 μM for 1 h at 72 h post transfection with a RNAi against NPC1L1. Data are means ± SD of three to four independent experiments for each compound tested.

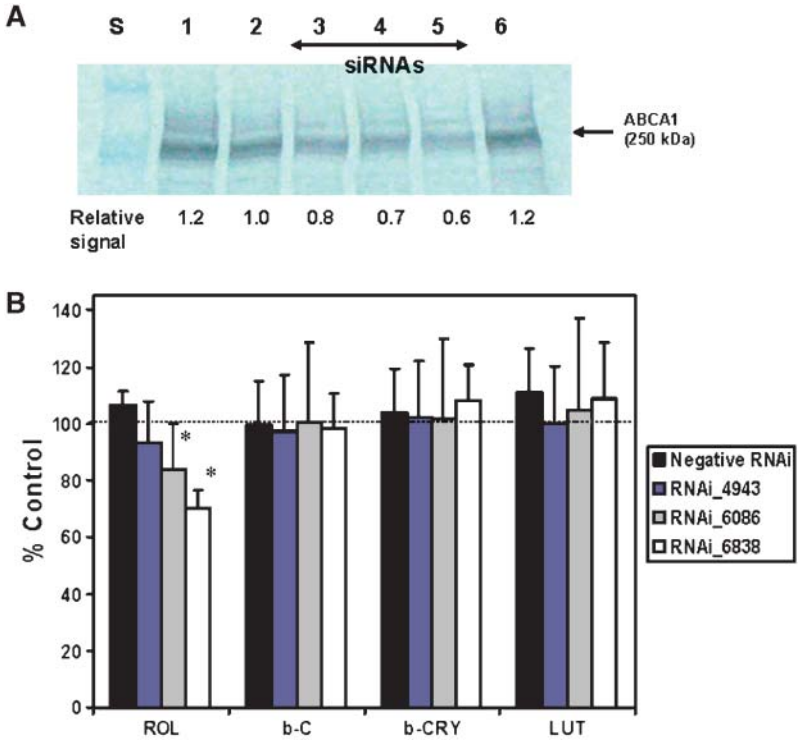

Fig. 8. Effects of the siRNA inhibition of ABCA1 expression on the net efflux of ROL, β-carotene (b-C), β-cryptoxanthin (b-CRY), or lutein (LUT) in Caco-2 cells. A: Immunoblots of ABCA1 expression (using 50 μg total protein/well) in cells treated under the following conditions: lane 1, scrambled RNAi; lane 2, LP2000 only; lane 3, RNAi_4943; lane 4, RNAi_6086; lane 5, RNAi_6838; lane 6, no treatment. Lane S represents protein standards (HiMark™ prestained standard from 55 to 460 kDa). B: Transport of ROL and carotenoids through Caco-2 cells and into the BM (expressed as the percentage of control cells treated with LP2000 only) after incubation of cells with ROL or a carotenoid at 2 μM for 4 h at 96 h post transfection with a RNAi against ABCA1. Data are means ± SD of three to four independent experiments for each compound tested. * $P < 0.05$ compared with the negative control.

gene silencing of NPC1L1 (70–90%) by the three siRNAs (RNAi_1831, RNAi_3832, and RNAi_4401) (Fig. 7A).

Knockdown of ABCA1 resulted in a reduction of ROL levels secreted into BM, with a significant effect for two of the three siRNAs used: ROL transport was reduced by 16% and 30%, respectively, for RNAi_6086 and RNAi_6838 treatments compared with the negative RNAi control treatment ($P < 0.01$) (Fig. 8B). The data show a clear linear relationship between the degree of ABCA1 knockdown by the three siRNAs (Fig. 8A) and the degree of inhibition in ROL efflux (Fig. 8B) ($R^2 = 0.977$, n = 3). Note here that cellular levels of ROL were not affected in these conditions. Finally, ABCA1 knockdown did not have any effect on the transport of the three carotenoids through Caco-2 cells.

DISCUSSION

Several previous studies have examined ROL transport in the human intestinal cell line Caco-2 (9, 25, 26). All have suggested that the process is facilitated, although the identity of the transporter(s) has remained unknown. After incubation with ROL at concentrations of 0.5–110 μM, the cellular uptake of ROL was directly proportional to the initial ROL concentration in the AM. However, the kinetics of ROL efflux into BM revealed two processes. ROL secretion was saturable at concentrations of <10 μM, suggesting a mediated transport out of the cell, and was linear at higher concentrations, suggesting passive diffusion. One interpretation of these data is that free ROL enters intestinal cells by simple diffusion, whereas its secretion may require a facilitated transport at physiological doses. Similar to our data, ROL was reported to be taken up by a saturable carrier-mediated process and a nonsaturable diffusion-dependent process in Caco-2 cells at physiological and pharmacological concentrations, respectively (9). However, in that study (9), because cells were grown on plates, it was not possible to differentiate between apical cellular uptake and basolateral secretion of ROL. This notion of facilitated ROL efflux is further supported by our experiments, in which glyburide, a known inhibitor of the ABCA1 transporter (27–29), caused marked inhibition of the efflux of free ROL into BM, but not of cellular uptake.

Of course, it is possible that cellular uptake at the apical membrane is also facilitated, but the rapid esterification of ROL after uptake makes it difficult to demonstrate kinetically that the transport is rate-limiting. Early studies using intestinal segments also suggested that unesterified ROL was taken up by protein-mediated facilitated diffusion and passive diffusion mechanisms at physiological (150 nM) and pharmacological (450–2,700 nM) concentrations, respectively (7, 8), but these systems also do not differentiate between apical uptake and basolateral efflux. Other evidence for the protein-mediated uptake of ROL has been presented using intestinal segments (11). Note that the kinetics of β-carotene transport through Caco-2 cells was different from those for ROL and that saturation of the cellular uptake of β-carotene was clearly observed at initial β-carotene concentrations of >10 μM (12).

There is much current interest in the role of membrane-bound lipid transporters in the cellular uptake and efflux of fat-soluble molecules. For example, three different membrane-bound proteins [cluster determinant 36 (CD36), membrane-bound fatty acid binding protein, and a fatty acid transport protein] that may be involved in fatty acid uptake have been identified (for review, see Refs. 30,

31). In the case of cholesterol, SR-BI, CD36, NPC1L1, and a variety of ABC transporters have been implicated in its uptake and/or efflux from various cells (32–36). It is possible that these or other proteins play a role in the transport of ROL across cell membranes. Defining the specific mechanisms of the transport of ROL (or other lipids) is complicated by the fact that, as indicated above, multiple mechanisms (both facilitated and passive) may exist in a single cell. An additional problem is that much of the work in this area relies on the use of membrane transporter inhibitors, and there is increasing evidence that some of these compounds inhibit multiple transporter types. Indeed, BLT4, a known inhibitor of SR-BI, was reported to block ABCA1-mediated cholesterol efflux, and reciprocally, glyburide, a known inhibitor of ABCA1, blocked SR-BI-mediated lipid transport (28).

Because of the uncertainty of the specificity of glyburide action for ABCA1, we also investigated the effect of the knockdown of several lipid transporters (ABCA1, SR-BI, and NPC1L1) by RNAi on ROL transport. Only ABCA1 inhibition affected ROL transport through Caco-2 cells, reinforcing the idea that ROL efflux is a facilitated process that involves the basolateral transporter ABCA1 but not the apical SR-BI and NPC1L1 transporters. It is interesting that siRNA treatments led to a linear relationship between the degree of knockdown (20–40% only) in ABCA1 protein expression and the degree of reduction (10–30%) in ROL efflux. This suggests that more complete inhibition of ABCA1 expression might lead to more complete inhibition of efflux (i.e., efflux facilitated by the transporter might be the major quantitative pathway for the transport of unesterified ROL through the cell).

The general concept that ROL is efficiently absorbed and quantitatively transported into lymph via CM may need reevaluation (37). Several studies have shown that the recovery of ingested ROL into lymph varied between 20% and 60% (6, 37, 38). The present data indicate that 16% of ROL passed through Caco-2 cell monolayers, and half of this was found as free ROL in the nonlipoprotein fraction. This suggests that the portal circulation could play a major role for in vivo ROL delivery to the liver. In vivo and in vitro studies support this idea. First, the amounts of absorbed ROL secreted into lymph and portal circulation were reported in vivo to be ~60% and ~30%, respectively (21). Second, oral supplements of ROL given to abetalipoproteinemia patients, who are unable to produce CM, resulted in partial recovery from symptoms of ROL deficiency (39). Finally, free ROL was transported across Caco-2 cells independently of the assembly and secretion of CM (26). When the nonlipoprotein fraction of BM was applied to a Sephadex G25M gel permeation column that separates large molecular weight solutes (e.g., proteins) from low molecular weight solutes, unesterified ROL (analyzed by HPLC) was recovered within the early, unretained, protein-containing fractions, whereas [^{14}C]glycerol was eluted in retained low molecular weight fractions (data not shown). These observations indicate that ROL secreted at the BM was probably bound to one or more proteins that remain to be characterized. Under our cell culture conditions, the amount of proteins in the medium could not influence ROL efflux, because the medium was protein (serum)-free on both the apical and basolateral sides. In vivo, we envisage that the process of unesterified ROL passage through intestinal cells involves ABCA1-mediated transport through the basolateral membrane and binding with HDL, retinol binding protein, or other proteins in the portal blood.

It was reported in vivo that REs were mostly present in CM and VLDL (40). Unlike TG, cholesteryl esters, and other lipids, REs are not present in intermediate density lipoproteins, low density lipoproteins, or high density lipoproteins (40). In accord with these previous reports, our data show that most REs secreted by Caco-2 cells were recovered in CM (65%) and VLDL (16%) fractions. We previously suggested that RE secretion by intestinal cells is a highly specific and regulated process that is dependent on the assembly and secretion of CM (26). RE secretion does not occur constitutively but is induced when cells can assemble and secrete CM. Thus, it appears that intestinal cells may have a specific mechanism for the targeting of REs to nascent CM (26). As discussed above, Caco-2 cells do not secrete REs under conditions simulating a fasting state. Supporting these ideas is the experiment in which cells were incubated with ROL for 16 h to accumulate cellular ROL and REs, followed by incubation with retinoid-free medium that was changed every 24 h. This resulted in the release of the free ROL but not the accumulated REs. This implies that only newly synthesized REs are incorporated into CM and that preformed REs cannot be used for CM assembly. Thus, the synthesis of REs and their incorporation into CM appear to be processes that work in concert.

Recent findings have suggested that carotenoid trafficking in the intestinal cell is a facilitated process that could involve more than one transporter (15). The present data indicate that, in contrast to ROL efflux from the intestinal cell, carotenoid efflux does not require the basolateral transporter ABCA1, supporting other evidence indicating that carotenoids mainly use the lymphatic (CM-associated) route in vivo. In contrast to cholesterol transport that was reported to be mediated via multiple pathways in Caco-2 cells (41), carotenoid transport appears to occur exclusively via the apolipoprotein B (apoB)-dependent pathway induced by OA but not via the apoB-independent pathway mediated by ABCA1. Note that, in the present study, Caco-2 cells were incubated without OA to promote the apoB-independent pathway, if present. Of the two apical transporters tested, NPC1L1 and SR-BI, only SR-BI inhibition affected carotenoid uptake by Caco-2 cells. This is in contrast to cholesterol transport, which may involve both NPC1L1 and SR-BI (42). The present data confirm the participation of SR-BI in the intestinal carotenoid trafficking, as suggested in recent reports by us and others (13–15).

We have shown that two independent pathways are available for the absorption of vitamin A in intestinal cells and provide new insights into the mechanisms involved. One pathway involves the resynthesis of REs from the newly absorbed unesterified ROL and their incorporation into

and secretion with CM. These data support the idea that the uptake of the unesterified ROL is rapid and not carrier-mediated (Figs. 1, 3). They also suggest that the processes of RE synthesis and incorporation into CM work in concert, in that preformed cellular REs were largely unavailable for subsequent incorporation into and secretion with CM (Fig. 4). The other pathway involves the facilitated efflux of unesterified ROL across the basolateral membrane (Fig. 3), a process that is inhibited by glyburide (Fig. 5) and that involves ABCA1 (Fig. 8). Although differing in details, these two processes are similar to those recently demonstrated for cholesterol by Iqbal, Anwar, and Hussain (41). Those authors showed that an "apoB-dependent" pathway involving CM synthesis is used for the secretion of cholesterol and cholesteryl ester in Caco-2 cells and that an "apoB-independent" pathway, mediated in part by ABCA1, is also involved in the basolateral secretion of unesterified cholesterol. These two independent pathways of vitamin A trafficking in intestinal cells would ensure the absorption of this essential micronutrient regardless of the amount and composition of the meal with which it is ingested.

In summary, the data presented here provide new information on several aspects of the mechanisms of the intestinal absorption of dietary provitamin A carotenoids and vitamin A itself. Intact carotenoids cross the mucosal membrane intact via a process facilitated by SR-BI, whereas the free ROL resulting from the hydrolysis of dietary RE appears to enter the cell without the facilitation of a protein transporter. Unesterified ROL in the mucosal cell thus arises from either direct uptake or partial cleavage of dietary provitamin A carotenoids in the mucosal cell. Some of this ROL is reesterified with long-chain fatty acids and incorporated directly into nascent CM along with intact carotenoids, newly synthesized TGs, and other lipid components of this apoB-containing intestinal lipoprotein. Thus, RE and intact carotenoids are only absorbed via CM and the lymphatic route. The free ROL in the intestinal cell can be transported directly across the basolateral membrane (presumably into the portal circulation) in a process that does not depend on CM assembly but that is mediated by ABCA1 and perhaps other transporters.

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