THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 6, Issue of February 7, pp. 3538–3543, 1997
Printed in U.S.A.

Bipolar Functional Expression of Transcobalamin II Receptor in Human Intestinal Epithelial Caco-2 Cells*

(Received for publication, September 5, 1996, and in revised form, November 21, 1996)

Santanu Bose‡, Shakuntla Seetharam§, Nancy M. Dahms‡, and Bellur Seetharam‡§¶

From the Departments of ‡Biochemistry and §Medicine, Medical College of Wisconsin and Veterans Administration Medical Center, Milwaukee, Wisconsin 53226

Transcobalamin II (TC II) receptor is expressed in the apical and basolateral membranes of human intestinal mucosa and in post-confluent human intestinal epithelial Caco-2 cells with a 6–7-fold enrichment in basolateral membranes. Caco-2 cells grown on culture inserts bound (at 5 °C) 30 and 180 fmol of the ligand, TC II-[^{57}Co]cobalamin (Cbl), to the apical and the basolateral surfaces, respectively. Within 5 h at 37 °C, all apically bound Cbl was internalized and subsequently transcytosed as TC II-Cbl. In contrast, all basolateral surface-bound Cbl was internalized and retained by the cells, but transferred from TC II to other cellular proteins. Chloroquine or leupeptin had no effect on the apical to basolateral transcytosis of either [^{57}Co]Cbl or ^{125}I-TC II. In contrast, following basolateral internalization of the ligand, both chloroquine and leupeptin inhibited the intracellular degradation of ^{125}I-TC II, which resulted in secretion of 60–65% of TC II-Cbl complex into the basolateral medium. When ^{125}I-TC II-Cbl was orally administered to rats, intact labeled TC II was detected in the portal blood 4 and 8 h later. These studies suggest that TC II-Cbl is processed when presented to the (*a*) apical/luminal side by a hitherto unrecognized non-lysosomal pathway in which both TC II and Cbl are transcytosed and (*b*) basolateral side by the lysosomal pathway in which TC II is degraded and the released Cbl is utilized.

The plasma transport of cobalamin (Cbl[1]; vitamin B_{12}) to all tissues/cells occurs bound to a plasma transporter, transcobalamin II (TC II), by receptor-mediated endocytosis (1) via transcobalamin II receptor (TC II-R). Recent studies (2) have shown that TC II-R is expressed as a non-covalent homodimer of molecular mass of 124 kDa in all human (2), rat (3), and rabbit (4) tissue plasma membranes. The plasma membrane expression of TC II-R is important for the tissue/cellular uptake of Cbl, since its functional inactivation *in vivo* by its circulatory antiserum causes intracellular deficiency of Cbl, which in turn results in the development of Cbl deficiency of the animal as a whole (4). Although TC II-R is expressed in the plasma membrane of all cells, its polarity of expression in epithelial cells is not known. Recent immunoblot studies (3) have revealed that in the rat kidney, TC II-R protein is expressed in both the isolated apical and basolateral membranes with an enrichment in the basolateral membranes by about 8-fold. However, how TC II-Cbl internalized via the TC II-R from either the apical or basolateral surface is processed is not known. Recent TC II-[^{57}Co]Cbl uptake studies (4) using filter-grown polarized Caco-2 cells have shown that [^{57}Co]Cbl taken up from the basolateral side in these cells was utilized as Cbl coenzymes, suggesting that these cells derive Cbl essential for their use from the basolateral side.

Despite these studies, the details of intracellular sorting of Cbl and TC II by a polarized epithelial cell are poorly understood. The present studies were undertaken to address the issues related to polarized expression and function of TC II-R in human intestinally derived Caco-2 cells, a well established cell model used extensively to study nutrient transport and general intestinal epithelial cell biology (5, 6).

The results of this study show that TC II-R is asymmetrically expressed in the ratio of 1:7 and 1:6.8 in the apical and the basolateral membranes of human intestinal mucosa and intestinally derived Caco-2 cells, respectively. Furthermore, when TC II-Cbl is presented on the apical side of Caco-2 cells or to the intestinal lumen of rats, TC II is transcytosed across the epithelial cell by a non-lysosomal pathway. In addition, these studies also demonstrate that when presented on the basolateral side, TC II is processed via the lysosomal pathway, resulting in the degradation of TC II and the utilization of intracellularly released Cbl as coenzymes.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals and reagents were obtained as indicated: [^{57}Co]cyanocobalamin (specific activity, 15 μCi/μg) and carrier-free Na^{125}I (Amersham Corp.), sulfosuccinimidobiotin (S-NHS-biotin) (Pierce), Millicell HA culture plate inserts (Millipore), cellulose nitrate membranes (Schleicher & Schuell), rabbit serum (Life Technologies, Inc.), human serum (Blood Center of Southeastern Wisconsin), chloroquine and leupeptin (Sigma), and Dulbecco's modified Eagle's medium and trypsin-EDTA (Life Technologies, Inc.). Human intestinal mucosa was obtained during autopsy of an unidentified donor from Frodetert Memorial Lutheran Hospital, Milwaukee, WI. Pure human TC II was a gift from the late Charles A. Hall (Nutrition Assessment Laboratories, VA Hospital, Albany, NY). Goat antiserum to rabbit TC II was a gift from Dr. Robert H. Allen (University of Colorado Health Science Center, Denver, CO).

Cell Culture—Caco-2 cells (passages 76–80) were grown in Dulbecco's modified Eagle's medium (25 mM glucose) supplemented with 20% heat-inactivated fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Confluent monolayers were subcultured every 7 days by treatment with 0.05% trypsin-EDTA in phosphate-buffered saline. In some experiments the cells were plated at a density of 2×10^6 cells on plastic (T-75 cm^2 flasks) and were harvested 3–12 days after plating. For the ligand uptake studies, cells were grown as epithelial layers by high density seeding (1.5×10^6 cells/filter) onto nitrocellulose membrane filter inserts (Millicell-HA, 30 mm diameter, 0.45 μM pore size). The formation and integrity of monolayers were assessed by the

* This work was supported by NIDDK Grant 50052 and VA Merit Grant (7816-01P) from the Department of Veterans Affairs awarded (to B. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom all correspondence should be addressed: MACC Fund Center, Rm. 6061, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Tel.: 414-456-4655; Fax: 414-259-1533.

[1] The abbreviations used are: Cbl, cobalamin (vitamin B_{12}); TC II, transcobalamin II; TC II-R, transcobalamin II receptor; IF, intrinsic factor; IFCR, intrinsic factor-cobalamin receptor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; S-NHS-biotin, sulfosuccinimidobiotin.

 This paper is available on line at http://www-jbc.stanford.edu/jbc/

This is an Open Access article under the CC BY license.

development of significant transepithelial resistance of 250–300 ohms/cm^2 over the resistance of filter alone. All resistance readings were measured with Millicell-ERS Voltohmeter (Millipore). Antiserum to human TC II-R (2) and human TC II (7) was prepared as described earlier. TC II free of transcobalamins I and III was partially purified from both rabbit and human serum according to Lindemans *et al.* (8) and used as ligand for uptake studies. Human TC II (5 μg) and streptavidin (50 μg) were each iodinated with 0.5 mCi of $Na^{125}I$ and IODOGEN, as recommended by the manufacturer (Pierce). The specific activity of iodinated TC II was 70–75 μCi/μg.

TC II-[^{57}Co]Cbl Binding and Uptake Studies in Filter-grown Caco-2 Cells—Post-confluent Caco-2 cells grown on culture inserts were incubated with human or rabbit TC II-[^{57}Co]Cbl (500 fmol) at 5 °C for 30 min either apically or basolaterally to determine the surface binding. After 30 min, the medium was removed, cells washed in cold medium, and the amount of TC II-[^{57}Co]Cbl bound to the surface membrane was determined by counting the radioactivity in scraped cells. The TC II-R-specific ligand binding was then calculated by subtracting the amount of ligand bound to the cell surfaces in the presence of TC II-R antiserum (5–20 μl) or that bound when the ligand was incubated at 5 °C in the presence of pH 5/EDTA buffer. In general, the nonspecific binding was less than 5% of the total ligand bound. Intracellular [^{57}Co]Cbl was determined by allowing the surface-bound ligand to internalize for 1 h at 37 °C. At the end of 1 h, the cells were scraped and counted for [^{57}Co]Cbl. In a separate set of filters, the surface-bound ligand from both surface membranes was incubated for 5 h at 37 °C to measure the amount of [^{57}Co]Cbl transported across the apical and the basolateral membranes. In some experiments prior to the addition of the ligand, the cells were incubated with either chloroquine (1 mg/ml) or leupeptin (1 mg/ml) added to both the apical and basolateral medium for 1 h. The lysosomotropic agents were also present throughout the 5 h period of incubation. During the apical ligand uptake studies, the basolateral surface was preincubated for 1 h at 4 °C with TC II-R antiserum (20 μl), washed with antiserum-free medium, and then warmed to 37 °C and the ligand was presented apically. Such a treatment was essential to ensure that TC II-[^{57}Co]Cbl that was transcytosed from the apical to the basolateral side did not re-enter the cell via basolateral TC II-R.

The radioactive Cbl present in the basolateral medium following internalization from the apical domain or from the basolateral domain of cells incubated with lysosomotropic agents was counted in a γ counter and then immunoprecipitated with polyclonal antiserum to either rabbit or human TC II. Briefly, 2 ml of the basolateral medium containing 23,000–150,000 dpm was treated with TC II antiserum (5–10 μl) and protein A-Sepharose (25–100 μl of 1:1 suspension in phosphate-buffered saline) and incubated for 18 h at 5 °C. The reaction mixture was centrifuged and radioactive Cbl immunoprecipitated was counted in a γ counter. Intracellular [^{57}Co]Cbl was determined by the immunoprecipitation of Triton X-100 (1%) extract of the cellular homogenates (500 μl of Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 0.1 mM PMSF) with antisera (5 μl) to either human or rabbit TC II as above.

When ^{125}I-TC II-Cbl was used as a ligand to study sorting of TC II from both surface domains, the filter-grown cells were incubated at 5 °C for 30 min with 350,000 dpm of ^{125}I-TC II-Cbl (specific activity 680 dpm/fmol) either on the apical or the basolateral side. The medium was removed, cells washed in cold medium, and then the surface-bound ligand was allowed to internalize for 1 h at 37 °C to measure the intracellular labeled TC II. Intracellular levels of ^{125}I-TC II was determined as follows; the cells were washed with pH 5.0.EDTA buffer to remove any surface radioactivity that might still be bound to the cell surfaces. Less than 1% of the radioactivity was removed from either surface by this treatment. The cells were then washed with Tris-HCl buffer, pH 7.4, containing 0.1 mM PMSF, scraped from filters, and homogenized in the same buffer (500 μl), and the radioactivity was counted in a γ counter. The cell pellet collected by centrifugation containing 15,000–100,000 dpm was then treated with non-reducing SDS-PAGE buffer. The liberated radioactivity (10,000–65,000 dpm) was subjected to SDS-PAGE (10%). In a separate set of filters, the surface-bound ^{125}I-TC II-Cbl was incubated for 5 h at 37 °C. The ^{125}I radioactivity present in the basolateral medium (2 ml) was counted in a γ counter. The medium was then treated with trichloroacetic acid (10%), and the precipitated radioactivity (>90%) was washed with acetone to remove trichloroacetic acid. The washed pellet was then treated with non-reducing SDS-PAGE buffer, and the liberated radioactivity was subjected to SDS-PAGE (10%).

SDS-PAGE Analysis and Immunoblotting—Isolated total, basolateral, and apical membranes from human ileal mucosa were subjected to non-reducing SDS-PAGE (7.5%) according to Laemmli (9). Separated proteins were electroblotted for 90 min to detect optimal levels of TC II-R dimer onto nitrocellulose membranes and probed with TC II-R antiserum as described before (2, 10).

Cell Surface Biotinylation—Biotinylation of Caco-2 cell surface proteins was carried out by adding S-NHS-biotin (0.5 mg/ml) to the apical and basolateral compartments of filter-grown monolayers (12-day growth) and was performed a total of three times for 30 min each at 4 °C in PBS containing 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$. The cells were then washed six times in PBS containing 0.1 mM $CaCl_2$ and $MgCl_2$, and 50 mM glycine (5 min each) at 4 °C. The cells were then harvested and extracted with PBS containing Triton X-100 (1%). The extract was immunoprecipitated with TC II-R antiserum (25 μl) and protein A-Sepharose (100 μl of 1:1 suspension in PBS). The washed immune pellet was subjected to non-reducing SDS-PAGE (7.5%), the bands were transferred to nitrocellulose membranes and probed with ^{125}I-streptavidin (3×10^7 dpm/blot), and the bands were visualized by autoradiography as described above.

In Vivo Transport of TC II in Rat—Six male adult rats (150 g) were orally administered with ^{125}I-TCII-Cbl (1×10^6 dpm of TC II, Cbl bound 1.5 pmol) using feeding tube as described earlier (11). Four and 8 h following the oral administration of the ligand, the rats were anesthetized, and blood was drawn from the portal vein between the intestine and the liver. Serum prepared from the whole blood drawn after 4 and 8 h of oral administration contained 50–75 $\times 10^3$ and 150–200 $\times 10^3$ dpm/ml, respectively. One fourth the radioactivity in each of the samples was subjected to non-reducing SDS-PAGE (10%). The gels were dried, and the radioactive bands were visualized by autoradiography at −70 °C using Kodak XAR-5 film. Similar results were obtained on SDS-PAGE using radioactive serum from each of the six rats used in the study.

Other Methods—Protein in all samples was determined according to Bradford (12). TC II-R activity in Caco-2 cell homogenate and isolated human intestinal membranes were determined using Triton X-100 (1%) extracts of these fractions by the DEAE-Sephadex method of Seligman and Allen (13). Total membranes from human ileal mucosa was obtained by centrifuging at 100,000 $\times g$ a 10% homogenate of the mucosa prepared in 10 mM potassium-phosphate buffer, pH 7.0, containing 0.25 M sucrose and 5 mM EDTA. The apical membranes from the human ileum was prepared by the Ca^{2+} aggregation method of Kessler *et al.* (14). The apical membrane was enriched for the apical markers, intrinsic factor-cobalamin receptor (10-fold), and alkaline phosphatase (17-fold) and contained <1% of the basolateral membrane marker, Na^+/K^+ ATPase, and other intracellular membrane components, NADPH-cytochrome *c* reductase and β-glucuronidase. The mucosal basolateral membrane was isolated by the method of Molitoris and Simon (15). TC II-R activity was enriched 8-fold and the basolateral marker Na^+/K^+ was enriched about 15-fold. The activity of apical markers, alkaline phosphatase, and IFCR, were present in amounts less than 1%.

RESULTS

Transcobalamin II Receptor Expression in Caco-2 Cells—In order to examine TC II-R activity in Caco-2 cells as a function of cellular proliferation and differentiation, its activity was determined in cells that were grown on plastic for 3–12 days (Fig. 1). Total TC II-R activity in the cellular homogenate rose by about 5-fold (from 11 to 49 pmol) from day 3 to day 12 in culture. When the activity was expressed in picomoles/mg of cellular protein, it rose by nearly 2.7-fold (2.7–7.2 pmol/mg of protein). These results demonstrated that TC II-R activity in these cells is regulated during their differentiation and that highest levels of its expression occurred around days 10–12 in culture after reaching confluence between days 5 and 6. In all further studies, post-confluent cells grown for 12 days were used.

Bipolar Expression of TC II-R in Human Intestinal Mucosa and Filter-grown Caco-2 Cells—Immunoblotting studies (3) using isolated apical and basolateral membranes from rat kidney and ligand binding studies using isolated rabbit intestinal surface membranes (4) have indicated that TC II-R protein and activity is enriched on the basolateral domain by nearly 8–10-fold. In order to confirm the bipolar asymmetric TC II-R protein and activity distribution between the apical and the basolateral surfaces in human intestine and in human intestinally derived Caco-2 cells, its activity and protein distribution were deter-

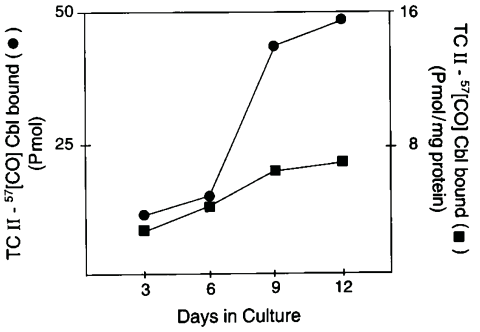

FIG. 1. **Transcobalamin II receptor activity during the growth and differentiation of Caco-2 cells.** Caco-2 cells grown on plastic for 3–12 days were harvested and homogenized in 10 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 0.1 mM PMSF. The homogenate was extracted with Triton X-100 (1%), and the supernatant was assayed for the binding of TC II-[^{57}Co]Cbl (2 pmol) according to Seligman and Allen (13). Each data point is the average of duplicate assays from two separate batch of cells grown for 3–12 days. In general the results varied by less than 5%.

FIG. 2. **Immunoblot analysis of total, apical, and basolateral membranes from human intestinal mucosa.** Total (*lane 1*), basolateral (*lane 2*), and apical (*lane 3*) membranes (50 μg of protein) were separated on non-reducing SDS-PAGE (7.5%), and the separated proteins were electroblotted onto nitrocellulose for 90 min and probed with antiserum to human placental TC II-R and ^{125}I-protein A. The bands were visualized by autoradiography.

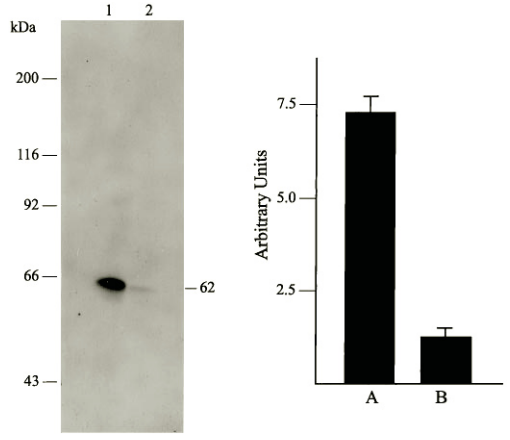

FIG. 3. **Domain-specific biotinylation of Caco-2 cells.** Post-confluent Caco-2 cells grown on Millipore culture inserts were biotinylated with S-NHS-biotin as described under "Experimental Procedures." *Left panel*, detection of immunoprecipitated biotinylated TC II-R separated on non-reducing SDS-PAGE (7.5%) using ^{125}I-streptavidin in basolateral (*lane 1*) and apical (*lane 2*) membranes. *Right panel*, the bands shown in the *left panel* were quantified from gels using biotinylated membranes from three separate filters. *A*, basolateral; *B*, apical.

mined by using isolated human intestinal surface membranes and filter-grown polarized Caco-2 cells. TC II-R activity in the human apical membranes was 1.4 pmol/mg of protein, which was about 7-fold less than the 11 pmol/mg of protein receptor activity noted in the basolateral membranes. Immunoblot analysis of isolated human intestinal total, apical, and basolateral membranes (Fig. 2) and domain-specific biotinylation of filter-grown Caco-2 cells were carried out (Fig. 3). Quantitation of these blots (Fig. 2) revealed that the 124-kDa dimer form of TC II-R was enriched by 8-fold in the total (*lane 1*) and 7-fold in the basolateral membranes (*lane 2*) relative to its level of expression in the apical membrane (*lane 3*). In the filter-grown Caco-2 cells, domain-specific biotinylation (Fig. 3, *left panel*) revealed TC II-R monomer of molecular mass of 62 kDa in both the basolateral (*lane 1*) and the apical (*lane 2*) cell surfaces. When the bands were quantitated (Fig. 3, *right panel*) for the image density, the receptor was enriched in the basolateral side (*A*) by nearly 6.8-fold relative to the apical side (*B*). TC II-[^{57}Co]Cbl binding to these surface membranes revealed that receptor-specific binding to the apical and basolateral membranes was 30 and 180 fmol/filter, respectively (Table I).

The absence of 124-kDa TC II-R dimer following domain-specific biotinylation of filter-grown Caco-2 cells is due to Triton X-100 extraction of the cells prior to treatment with TC II-R antiserum. Previously (2) we have shown that treatment of native membranes with Triton X-100 results in the total conversion of TC II-R dimer of molecular mass of 124 kDa into the monomer of molecular mass of 62 kDa. With the human intestinal membranes, immunoblotting (Fig. 2) did reveal very faint 62-kDa TC II-R monomers in the total (*lane 1*) and the basolateral membranes (*lane 2*). Previously we have shown that TC II-R monomer levels are only 10% of the TC II-R dimer levels in any given tissue (3) and that the monomers are lost during 90 min electroblotting (10). These studies have shown that apical expression of TC II-R activity/protein is a property of not only intact intestinal mucosa but also of cultured intestinally derived Caco-2 cells. In order to examine whether TC II-R expressed in both surface domains is functional in mediating endocytosis, we used filter-grown Caco-2 cells to study binding and uptake of the ligand.

Cellular Sorting of [^{57}Co]Cbl Bound to TC II—Filter-grown cells were presented with the ligand human TC II-[^{57}Co]Cbl on either the apical or basolateral cell surfaces. The ligand binding to cell surfaces at 5 °C revealed a basolateral binding of ~ 180 fmol/filter, which was nearly 6-fold higher than the binding of ~ 30 fmol/filter noted on the apical side (Table I). The specificity of ligand binding was confirmed when preincubation of cells with TC II-R antiserum on either side completely abolished ligand binding (data not shown). When the cells were warmed to 37 °C to allow for internalization of the ligand, within 60 min all the labeled Cbl had left both the apical and basolateral cell surfaces and 100% of [^{57}Co]Cbl was found inside the cell (Table I). However, 5 h following internalization, nearly 100% of Cbl internalized from the basolateral side was still present inside the cell while, greater than 95% of Cbl internalized from the apical side had exited the cell and was found transcytosed to the basolateral medium. When the cells were preincubated with lysosomotropic agents, either chloroquine or leupeptin, the amount of [^{57}Co]Cbl that was transcytosed to the basolateral side following apical internalization of TC II-[^{57}Co]Cbl was not inhibited. In contrast, lysosomotropic agents inhibited the intracellular [^{57}Co]Cbl levels by nearly 65–70% following its basolateral entry and the inhibition was accompanied by increased [^{57}Co]Cbl levels in the basolateral medium. Both in the absence and in the presence of lysosomo-

TABLE I
Binding and internalization of TC II-[^{57}Co]Cb from Caco-2 cell surface membranes
The values reported here are mean ± S.D. from 8 filters used for each experiment. Other details are provided under "Experimental Procedures."

Side of ligand presentation	Surface-bound (5 °C/30 min)	Internalized (37 °C/1 h)	Intracellular (37 °C/5 h)	Basolateral medium (37 °C/5 h)
		[^{57}Co]Cbl (fmol/filter)		
Apical	32 ± 2	30 ± 3	5 ± 1	27 ± 5
+ Chloroquine	32 ± 3	31 ± 2	4 ± 1	28 ± 4
+ Leupeptin	33 ± 3	31 ± 2	3 ± 1	28 ± 3
Basolateral	175 ± 8	180 ± 5	178 ± 8	0
+ Chloroquine	176 ± 7	172 ± 7	65 ± 7	124 ± 7
+ Leupeptin	179 ± 9	175 ± 9	55 ± 5	120 ± 5

tropic inhibitors, basolaterally internalized Cbl was never transcytosed to the apical side (data not shown). These results suggested that apically internalized [^{57}Co]Cbl bound to TC II was transcytosed by a non-lysosomal pathway, while the basolaterally internalized [^{57}Co]Cbl was retained in the cell, and that the lysosomotropic agents chloroquine and leupeptin inhibited the cellular retention of [^{57}Co]Cbl.

Immunoprecipitation of basolateral [^{57}Co]Cbl with antiserum to human TC II revealed (Table II) that all the Cbl radioactivity (~28 fmol) present in the basolateral medium following apical internalization of human TC II-[^{57}Co]Cbl in the presence and absence of lysosomal inhibitors was immunoprecipitated. In contrast, >90% of intracellular [^{57}Co]Cbl (~172–180 fmol) following internalization of TC II-[^{57}Co]Cbl from the basolateral side could not be immunoprecipitated with antiserum to human TC II when the cells were incubated in the absence of lysosomal inhibitors (Table II). However, in the presence of lysosomal inhibitors, [^{57}Co]Cbl that was in the cell (55–65 fmol) and in the basolateral medium (120–124 fmol) was completely precipitated with human TC II antiserum (Table II).

Although the apically presented [^{57}Co]Cbl was completely transcytosed and was bound to TC II, it is not known whether the source of basolateral TC II is the internalizing exogenous TC II or the endogenous Caco-2 cell TC II to which Cbl is transferred prior to transcytosis and following degradation of the exogenous internalizing TC II. To address this issue, the following experiments were carried out. Our initial transcytosis studies using [^{57}Co]Cbl bound to rabbit TC II revealed that all of labeled Cbl in the basolateral medium could be precipitated with antiserum to rabbit TC II but not with antiserum to human TC II (data not shown). Native TC II complexed with [^{57}Co] Cbl from these two species can only be precipitated with their respective antiserum but not with antiserum raised to the other species (16). Thus, this observation suggested that the source of TC II in the basolateral medium following apical internalization of TC II-Cbl is the exogenous TC II. In order to prove this directly, further transcytosis studies were carried out using ^{125}I-TC II-Cbl.

TC II Is Processed by Both Lysosomal and Non-lysosomal Pathways in Caco-2 Cells—When ^{125}I-TC II-Cbl was presented on the apical side (Fig. 4, *left panel*), intact TC II could be detected inside the cell within 1 h, both in the absence (*lane 3*) and the presence of lysosomotropic inhibitors chloroquine (*lane 1*) or leupeptin (*lane 2*). When the ligand was presented basolaterally, within 1 h, no labeled TC II could be detected in the cell (*lane 7*) in the absence of lysosomotropic agents. However, intact TC II could be detected in the cells when the cells were incubated with either chloroquine (*lane 5*) or leupeptin (*lane 6*). Following 5 h of internalization, intact labeled TC II could be seen transcytosed to the basolateral medium both in the absence (Fig. 4, *right panel*, *lane 3*) or the presence of chloroquine (*lane 1*) or leupeptin (*lane 2*) when ^{125}I-TC II-Cbl was internalized from the apical side. In contrast, when the ligand was presented to the basolateral side, intact TC II could be visualized in the basolateral medium only when the cells were exposed to chloroquine (*lane 5*) or leupeptin (*lane 6*). In the absence of treatment of cells with lysosomotropic agents, the basolateral media contained degraded TC II of molecular mass of 28 kDa (*lane 7*), 15 kDa smaller than the native labeled TC II of molecular mass of 43 kDa (*lane 0*). The bands shown in the *left panel* (*lanes 0* and *4*) and the *right panel* (*lanes 0* and *4*) represent unincubated ^{125}I-TC II run on the same gels to match the intensities of ^{125}I-TC II present in the cells (*left panel*) or in the basolateral medium (*right panel*). These results indicated that Caco-2 cells internalize TC II-Cbl from both plasma membrane domains but process them differently. While the apically internalized TC II-Cbl is transcytosed intact to the basolateral medium, the basolaterally internalized TC II-Cbl is processed by the lysosomes involving the degradation of TC II and the liberation of Cbl to be utilized by the cell.

TABLE II
Immunoprecipitation of intracellular and basolateral [^{57}Co]Cbl with human TC II antiserum
The results shown represent mean ± S.D. from 8 separate filters for each experiment. Other details of cell extraction and immunoprecipitation are provided under "Experimental Procedures."

Fraction	[^{57}Co]Cbl immunoprecipitated		
	Control	Chloroquine	Leupeptin
	fmol/filter		
Apical ligand presentation			
Basolateral medium	28 ± 2	27 ± 2	28 ± 3
Basolateral ligand presentation			
Cellular extract	5 ± 1	65 ± 5	55 ± 7
Basolateral medium	0	124 ± 5	128 ± 8

Apical TC II-R Is Functional in Intact Intestine—Since Caco-2 cells are derived from human colon tumors, the results obtained, the apical to basolateral transcytosis of TC II-Cbl, may be an artifact of culture and does not reflect situations in an intact intestine. In order to test whether the rat intestinal apical TC II-R is functional in the internalization of TC II-Cbl, the ligand, ^{125}I-TC II-Cbl, was orally administered to rats. Following 4 h of oral administration, intact 43-kDa labeled TC II was detected (Fig. 5) in portal blood. Within 8 h the intensity of the band had increased, but a small amount of degraded TC II of molecular mass of 35 and 28 kDa could be detected. These results show that *in vivo*, TC II-R expressed on the luminal side is able to mediate the transcytosis of intact TC II into systemic circulation.

DISCUSSION

In the current studies we have used human intestinally derived epithelial Caco-2 cells to study TC II-mediated transport of Cbl. Caco-2 cells are a well established cell model system, and because of their high degree of differentiation they have been used extensively to study the biosynthesis and polarized delivery of functional proteins of brush border (17). TC

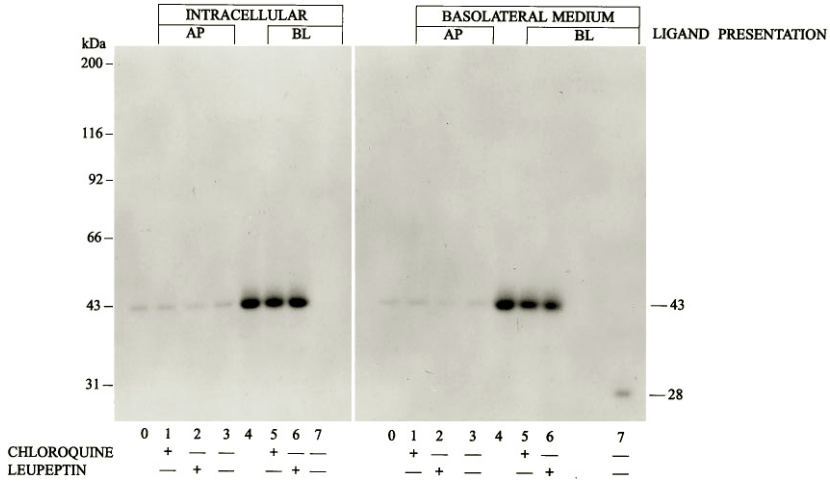

FIG. 4. **SDS-PAGE analysis of intracellular and basolateral ^{125}I-TC II-Cbl following internalization from the apical and basolateral sides of filter-grown Caco-2 cells.** *Left panel*, intracellular ^{125}I-radioactivity following 1 h of internalization (37 °C) from the apical (*lanes 1–3*) and the basolateral (*lanes 5–7*) sides. Prior to the presentation of the ligand on either side the cells were preincubated with chloroquine (1 mg/ml, *lanes 1* and *5*), leupeptin (1 mg/ml, *lanes 2* and *6*), or nothing (*lanes 3* and *7*). Bands shown in *lanes 0* and *lanes 4* represent 10,000 and 65,000 dpm of unincubated ^{125}I-TC II-Cbl. *Right panel*, basolateral medium ^{125}I-radioactivity following internalization (5 h at 37 °C) from the apical (*lanes 1–3*) and basolateral (*lanes 5–7*) sides. Other details are exactly as shown for the lanes in the *left panel*.

FIG. 5. **SDS-PAGE analysis of ^{125}I-radioactivity in rat serum.** Serum was prepared from rat blood drawn following 4 and 8 h of oral administration of ^{125}I-TC II-Cbl to rats. Other details are provided under "Experimental Procedures."

II-R total and specific activities (Fig. 1), like those of IFCR (7), alkaline phosphatase (18), and sucrase (17), rose as a function of differentiation of these cells. The bipolar expression and the basolateral enrichment of TC II-R noted in these cells (Fig. 3) are not artifacts of culture since isolated surface membranes from intact intestinal mucosa also exhibited these properties (Fig. 2).

Recent (4) studies from our laboratory have shown that TC II-[^{57}Co]Cbl internalized from the basolateral side of human intestinal epithelial Caco-2 cells is processed, releasing Cbl to be utilized as coenzymes by these cells. In the current studies we show that, following basolateral uptake of TC II-[^{57}Co]Cbl, TC II is degraded by lysosomal enzymes, facilitating the liberated free Cbl to be utilized by the cells. Under normal physiological conditions, the dietary Cbl present on the lumenal side bound to gastric intrinsic factor is transcytosed via IFCR in both the intact intestine (11, 19) and in Caco-2 cells (7, 20, 21) and other polarized epithelial cells (22–25). During intrinsic factor-mediated apical to basolateral transcytosis of Cbl, intrinsic factor is degraded by leupeptin-sensitive protease and Cbl is bound to transcobalamin II prior to its exit on the basolateral side (23–25). Since all the Cbl internalized bound to IF from the apical side (luminal) of polarized epithelial cells is eventually transcytosed, these cells must obtain Cbl from endogenous sources, and our current and recent (4) studies demonstrate that the basolateral TC II-R facing the circulation may facilitate such an uptake from the circulation.

The results of the current studies have also shown that the apical TC II-R expressed in Caco-cells (Table I and Fig. 4) and in the rat intestine (Fig. 5) is functional in mediating endocytosis of TC II-Cbl. The functional significance of apical endocytosis of TC II-Cbl and its eventual transcytosis by the nonlysosomal pathway in the gastrointestinal absorption of dietary/biliary Cbl is not known. It is highly unlikely that Cbl transport bound to TC II occurs physiologically bypassing the well accepted IF/IFCR pathway of Cbl transport (26) for the following two reasons. 1) TC II, the ligand has never been detected in the gastrointestinal lumen despite the recent finding of relatively high levels of TC II mRNA in human pancreas (27). However, it is not known whether TC II like IF in some species (28) is secreted from the pancreas and mediate luminal uptake of Cbl via TC II-R. 2) patients with inherited disorders (29, 30) of IF or IFCR develop Cbl deficiency, suggesting that IF/IFCR-mediated Cbl transport system is the only physiologically operational intestinal uptake system for Cbl transport in man. Despite its lack of importance in the normal uptake of dietary Cbl, it is possible that apical TC II-R can mediate uptake of TC II-Cbl when presented orally, particularly in patients who malabsorb Cbl due to several inherited disorders (29, 30) or to surgical procedures such as gastrectomy and ileal resection (31). A child who malabsorbed Cbl due to inherited TC II deficiency responded well in Schilling test (32) (which measures Cbl absorption) when the child was orally fed with Cbl complexed to TC II. Further studies are needed to test the usefulness of the apical uptake of TC II-Cbl if any, in vitamin B_{12} absorption disorders.

Based on previous (7, 20–25) and the current studies, several interesting questions arise regarding the mechanism of Cbl sorting when internalized bound to IF or TC II in a polarized epithelial cell. These include the following. (*a*) how are these cells able to distinguish between Cbl internalized apically

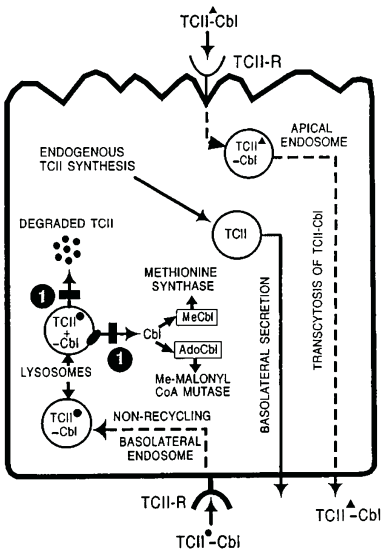

FIG. 6. **Proposed sorting pathways of TC II and Cbl in Caco-2 cells.** Proposed pathways are indicated by *broken lines*. More established pathways are indicated by *full lines*. TC II internalized bound to Cbl from the apical side (▲) and the basolateral side (●) is shown. The role of lysosomotropic agents chloroquine and leupeptin on the degradation of basolaterally derived TC II, and the resulting transport out of the lysosomes into the cytosol for conversion to MeCbl and AdoCbl is indicated by *Block 1*. The lysosomal transporter (35), which is thought to a play a role in the transfer of free Cbl from the lysosomes to the cytosol, is indicated by a *dark oval*.

bound to IF or basolaterally bound to TC II to either export Cbl (transcytose) out of the cell or import it into the cell, respectively? In addition, what vesicular fusion events favor lysosomal degradation of both IF and TC II when internalized from the opposite side of these cells? (*b*) how do these cells process the same ligand TC II, by both the non-lysosomal and lysosomal pathways following its internalization from the apical and the basolateral domains, respectively? The cellular mechanism/s by which Caco-2 cells are able to mediate these sorting events is not known. Possible explanations for the lysosomal processing of IF or the non-lysosomal processing of TC II during apical to basolateral transcytosis of Cbl may be that it is due to (*a*) endocytosis by two separate receptors, IFCR and TC II-R, respectively, or (*b*) due to the differences in the nature of the internalizing ligands, glycoprotein (IF) (33) *versus* non-glycoprotein (TC II) (34).

One observation of interest in Caco-2 cells is that endogenously synthesized (7) or exogenously derived TC II from the apical domain (Fig. 4) or exogenous TC II derived from the basolateral side whose lysosomal degradation is inhibited is secreted to the basolateral side. Even the degradation product of basolaterally derived TC II is secreted to the basolateral side. Thus, it seems that TC II, irrespective of its initial vesicular location in the cell, is targeted for basolateral secretion in these cells and perhaps in the absorptive enterocytes. The basolateral exit of TC II-Cbl in Caco-2 cells does not need TC II-R as the apical to basolateral transcytosis of TC II-Cbl occurred even when the basolateral TC II-R was inactivated with its antiserum during these experiments to ensure that the transcytosed TC II-Cbl does not re-enter the cell. The processing of TC II by two separate pathways depending on the side of internalization noted in this study may be related to the differences in the nature of the initial endocytic vesicles derived from the opposite sides of the cell or to differences in the later fusion events. Further studies are needed to define the nature of transporting vesicles and vesicular fusion events involved in the segregation of TC II taken up from the opposite side of these cells.

Based on our previous (4) and current findings, we propose a model (Fig. 6) for the TC II-mediated vectorial movement of Cbl into and out of polarized epithelial cells. Based on this model, polarized epithelial cells such as absorptive enterocytes derive Cbl for their intracellular utilization as Cbl coenzymes from the circulation bound to plasma TC II via TC II-R expressed in the basolateral membranes. The basolaterally derived TC II-Cbl is processed via lysosomes and free Cbl formed due to TC II degradation is transported out of the lysosomes for conversion to and utilization as Cbl coenzymes. In contrast, apical TC II-R when presented with TC II-Cbl is able to transcytose both Cbl and TC II across the cell. Further studies are needed to test the validity of this proposal in other intestinal and renal polarized epithelial cells.

REFERENCES

1. Youngdahl-Turner, P., Rosenberg, L. E., and Allen, R. H. (1978) *J. Clin. Invest.* **61,** 133–141
2. Bose, S., Seetharam, S., and Seetharam, B. (1995) *J. Biol. Chem.* **270,** 8152–8157
3. Bose, S., Seetharam, S., Hammond, T. G., and Seetharam, B. (1995) *Biochem. J.* **310,** 923–929
4. Bose, S., Komorowski, R., Seetharam, S., Gilfix, B., Rosenblatt, D. S., and Seetharam, B. (1996) *J. Biol. Chem.* **271,** 4195–4200
5. Rindler, M. J and Hoops, T. C. (1994) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed) 3rd Ed., pp. 1623–1645, Raven Press, New York
6. Rodriguez-Boulan, E., and Powell, S. K. (1992) *Annu. Rev. Cell. Biol.* **8,** 258–288
7. Ramanujam, K. S., Seetharam, S., Ramasamy, M., and Seetharam, B. (1991) *Am. J. Physiol.* **260,** G416–G422
8. Lindemans, J., Kroes, A. C. M., Geel, J. V., Schoester, M., and Abels, J. (1986) *J. Exp. Cell. Res.* **184,** 449–460
9. Laemmli, U. K. (1970) *Nature* **227,** 680–685
10. Bose, S., Feix, J., Seetharam, S., and Seetharam, B. (1996) *J. Biol. Chem.* **271,** 11718–11725
11. Ramasamy, M., Alpers, D. H., Tiruppathi, C., and Seetharam, B. (1989) *Am. J. Physiol.* **257,** G791–G797
12. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
13. Seligman, P. A., and Allen, R. H. (1978) *J. Biol. Chem.* **253,** 1766–1772
14. Kessler, M., Acuto, O., Storelli, C., Murer, M., and Semenza, G. (1978) *Biochim. Biophys. Acta* **506,** 136–154
15. Molitoris, B. A., and Simon, F. R. (1985) *J. Membr. Biol.* **83,** 207–215
16. Ramanujam, K. S., Seetharam, S., and Seetharam, B. (1991) *Biochem. Biophys. Res. Commun.* **179,** 543–550
17. Zweibaum, A., Labruthe, M., Grasset, E., and Louvard, D. (1991) in *Handbook of Physiology, Section 6: The Gastrointestinal System* (Field, M., and Frizzell, R. A., eds) Vol. IV, pp. 223–255, American Physiological Society, Bethesda, MD
18. Pinto, M., Robin-Leon, S., Appay, M. D., Kedinger, M., Triadou, E., Dussaulex, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J., and Zweibaum, A. (1983) *Biol. Cell.* **47,** 323–330
19. Robertson, J. A., and Gallagher, N. D. (1985) *Gastroenterology* **88,** 908–912
20. Dix, C. J., Hassan, I. F., Obray, H. Y., Shah, R., and Wilson, G. (1990) *Gastroenterology* **98,** 1272–1278
21. Dan, N., and Cutler, D. F. (1994) *J. Biol. Chem.* **269,** 18849–18855
22. Gueant, J. L., Masson, C., Schohn, H., Girr, M., Saunier, M., and Nicolas, J. P. (1992) *FEBS Lett.* **297,** 229–232
23. Gordon, M. M., Howard, T., Becich, M. J., and Alpers, D. H. (1995) *Am. J. Physiol.* **268,** G33–G40
24. Ramanujam, K. S., Seetharam, S., Dahms, N., and Seetharam, B. (1991) *J. Biol. Chem.* **266,** 13135–13140
25. Ramanujam, K. S., Seetharam, S., and Seetharam, B. (1992) *Biochem. Biophys. Res. Commun.* **182,** 439–446
26. Seetharam, B. (1994) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed) 3rd Ed., pp. 1997–2026, Raven Press, New York
27. Li, N., Seetharam, S., Rosenblatt, D. S., and Seetharam, B. (1994) *Biochem. J.* **310,** 585–590
28. Batt, R. M., and Horadagoda, N. U. (1989) *Am. J. Physiol.* **257,** G9344–G9349
29. Cooper, B. A., and Rosenblatt, D. S. (1987) *Annu. Rev. Nutr.* **7,** 291–296
30. Fenton, W. A., and Rosenberg, L. E. (1989) in *Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., Valley, D., eds) 2nd Ed., pp. 2065–2082, McGraw Hill Information Service Company, New York
31. Seetharam, B., and Alpers, D. H. (1982) *Annu. Rev. Nutr.* **2,** 343–369
32. Barshop, B. A., Wolf, J., Nyham, W. L., Alice, Y. Prodanos, C., Jones, G., Sweetman, L., Leslie, J., Holm, J., Green, R., Jacobsen, D. W., Cooper, B. A., and Rosenblatt, D. S. (1990) *Am. J. Med. Genet.* **35,** 222–228
33. Allen, R. H., and Mehlman, C. S. (1973) *J. Biol. Chem.* **248,** 3660–3669
34. Allen, R. H., and Majerus, P. W. (1972) *J. Biol. Chem.* **247,** 7709–7717
35. Idriss, J. M., and Jonas, A. J. (1991) *J. Biol. Chem.* **266,** 9438–9441