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

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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-[57Co]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 [57Co]Cbl or 125I-TC II. In contrast, following basolateral internalization of the ligand, both chloroquine and leupeptin inhibited the intracellular degradation of 125I-TC II, which resulted in secretion of 60–65% of TC II-Cbl complex into the basolateral medium. When 125I-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); vitamin B12 occurs to all tissues/cells 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-[57Co]Cbl uptake studies (4) using filter-grown polarized Caco-2 cells have shown that [57Co]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: [57Co]cyanocobalamin (specific activity, 15 μCi/μg) and carrier-free Na125I (Amersham Corp.), sulfosuccinimidobiotin (S-NHS-biotin) (Pierce), Millipore 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 Froedert Memorial Lutheran Hospital, Milwaukee, WI. Pure human TC II was a gift from the late Charles A. Hall (Nutrition Assessment 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% CO2. 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 × 104 cells on plastic (T-75 cm2 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 × 106 cells/filter) onto nitrocellulose membrane filter inserts (Millipore-HA, 30 mm diameter, 0.45 μm pore size). The formation and integrity of monolayers were assessed by the
development of significant transepithelial resistance of 250–300 ohms/cm² over the resistance of filter alone. All resistance readings were measured with Millicell-ERS Volthometer (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 the serum by affinity chromatography on a gel containing human serum albumin (HSA) ligand and used as ligand for uptake studies. Human TC II (5 μg) and streptavidin (50 μg) were each iodinated with 0.5 mCi of Na¹²⁵I and IODOGEN, as recommended by the manufacturer (Pierce). The specific activity of iodinated TC II was 70–75 μCi/μg.

**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.

**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 × g for 15,000–100,000 dpm and less than 1% was recovered in the supernatant. The apical membranes from the human ileum were prepared by the Ca²⁺ aggregetion 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%.

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Transcobalamin II receptor expression in Caco-2 cells

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, 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 medium was abolished (data not shown). When the cells were treated with chloroquine or leupeptin, the amount of $^{57}$Co/Cbl that was transcytosed to the basolateral medium was abolished (data not shown).

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 batches of cells grown for 3–12 days. In general, the results varied by less than 5%.

The absence of 124-kDa TC II-R dimer following domain-specific biotinylation of filter-grown Caco-2 cells was 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) revealed very faint 62-kDa TC II-R monomer 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.

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

Immunoprecipitation of basolateral $[^{57}\text{Co}]$Cbl with antiserum to human TC II revealed (Table II) that all of labeled Cbl in the basolateral medium could 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, $[^{57}\text{Co}]$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 $[^{57}\text{Co}]$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 $[^{57}\text{Co}]$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 $[^{57}\text{Co}]$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.

To prove this directly, further transcytosis studies were carried out using $^{125}$I-TC II-Cbl.

**Table I**

| 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) |
|----------------------------|-----------------------------|--------------------------|--------------------------|-------------------------------|
| Apical                     |                             |                          |                          |                               |
| + Chloroquine              | 32 ± 2                      | 30 ± 3                   | 5 ± 1                    | 27 ± 5                       |
| + Leupeptin                | 32 ± 3                      | 31 ± 2                   | 4 ± 1                    | 28 ± 4                       |
| Basolateral                | 33 ± 3                      | 31 ± 2                   | 3 ± 1                    | 28 ± 3                       |
| + Chloroquine              | 175 ± 8                     | 180 ± 5                  | 178 ± 8                  | 124 ± 7                      |
| + Leupeptin                | 176 ± 7                     | 172 ± 7                  | 65 ± 7                   |                                |

**Table II**

| Fraction                  | $[^{57}\text{Co}]$Cbl immunoprecipitated fmol/filter |
|---------------------------|------------------------------------------------------|
| Apical ligand presentation|                                             |
| Basolateral medium        | 28 ± 2                                                |
| Basolateral ligand         | 27 ± 2                                                |
| Cellular extract           | 5 ± 1                                                 |
| Basolateral medium         | 124 ± 5                                               |

**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
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-[57Co]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-[57Co]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 luminal 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 non-lysosomal 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 mediates 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 B12 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...
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 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 antisera. When 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 transport 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.

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