Transcobalamin II synthesized in the intestinal villi facilitates transfer of cobalamin to the portal blood

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Gastrointest. Liver Physiol. 40: G161–G166, 1999.—This study was designed to identify the cellular component of the intestinal villus where transcobalamin II (TCII) is synthesized, because this protein provides an essential function in the intestinal absorption of vitamin B₁₂ (cobalamin, Cbl). When a segment of proximal or distal small intestine of the guinea pig is cultured in medium containing [⁵⁷Co]Cbl, TCII-[⁵⁷Co]Cbl appears within 15 min. Northern blot analysis of RNA from both proximal and distal small intestine identified the TCII transcript. In situ hybridization of the distal ileum with [³⁵S]-labeled TCII antisense transcript localized grains predominantly in crypts and in the lower third and central core of the villi. Grains were also evident at the base of the enterocytes in close apposition with the vascular network, whereas few grains appeared in the apical region of the columnar cells. This study provides evidence that TCII is constitutively expressed in the intestinal villi where vascular endothelium is abundant. In the distal ileum, where the intrinsic factor (IF) receptor is expressed, after uptake of IF-Cbl and the subsequent binding of free Cbl to TCII synthesized in the villi, the TCII-Cbl complex enters the microcirculation and passes into the portal blood.

intrinsic factor receptor; vitamin B₁₂; in situ hybridization; ileal cultures; endothelium

THE PHYSIOLOGICAL PROCESS of cobalamin (Cbl) absorption is mediated by intrinsic factor (IF), a mucoprotein that, in humans, is secreted primarily by the parietal cells in the stomach, and a receptor on the microvillus membrane of the distal ileum that binds and internalizes the IF-Cbl complex (10, 20). The absorbed Cbl appears in the blood bound to transcobalamin II (TCII), a 43-kDa plasma protein (24) that distributes the absorbed vitamin to all tissues where specific receptors on the cell membrane bind and internalize the TCII-Cbl complex by endocytosis (9). A deficiency of IF or TCII, or mutations of their respective genes, results in Cbl deficiency that is usually characterized clinically by hematologic (13) and/or neurological disorders (14). Interestingly, malabsorption of Cbl has also been observed in patients with congenital absence of TCII (4, 13), indicating that TCII functions in the transfer of Cbl through the ileal mucosa.

Previous studies in guinea pigs (28) and humans (6) have shown that the absorbed Cbl appears bound to TCII that is not derived from the pool of TCII in the blood. The present study was undertaken to identify the origin of TCII within the intestinal villi of the distal ileum, because expression of TCII in the small intestine appears essential for the absorption of dietary Cbl.

METHODS

Materials. White female Hartley guinea pigs (200–250 g) were obtained from Charles River (Wilmington, MA); Trowell’s medium was from GIBCO (Grand Island, NY); cyanocobalamin, bovine hemoglobin, and activated charcoal came from Sigma (St. Louis, MO); [⁵⁷Co]cyanocobalamin was from Johnson and Johnson; QUSO G35 microfine silica came from Degusa (Teterboro, NJ); and Sephacryl S-200 was from Pharmacia Biotech (Piscataway, NJ). All other reagents were of analytical grade.

In vitro culture studies. Guinea pigs used in this study were fasted for 72 h before the experiment but were provided with water ad libitum. Animals were anesthetized by Metofane inhalation and were maintained on this anesthetic during the entire surgical procedure. The ileum was exposed by opening the abdominal cavity, and ligatures were applied just above the cecum and ~20 cm proximal to the ileocecal junction. A small incision was made above the lower ligature, and the lumen was flushed by injecting ~20 ml of saline below the upper ligature with a needle and syringe. This segment of the ileum was then removed and placed in Trowell’s medium at 4°C.

The ileal segments were set up in culture essentially as described by Browning and Trier (3). Briefly, the ileum was divided into ~2-cm segments, and each segment was cut open longitudinally and placed on stainless steel grids with the lumen facing up in 6-well culture plates containing 2 ml Trowell’s medium with either no Cbl or 1 ng of [⁵⁷Co]Cbl and without supplementing with FBS. To assay for protein-bound [⁵⁷Co]Cbl in the postculture medium, 1 ml of the medium was mixed with 0.5 ml of hemoglobin-coated charcoal (11) to adsorb free Cbl. The supernatant fraction was mixed with 100 µl of QUSO G35 (30 mg/ml) (15) to adsorb TCII-bound [⁵⁷Co]Cbl. For the determination of unsaturated TCII (apo-TCII) in the culture medium, one 0.5-ml aliquot was incubated with 20 pg and another with 100 pg of [⁵⁷Co]Cbl for 30 min followed by separation of free Cbl and TCII-Cbl as described above. The TCII-Cbl complex in the culture medium was also identified by size-exclusion chromatography on Sephacryl S-200 as previously described (23).

Directional transport of TCII in the ileum. A fresh ileal segment was prepared as described above, rinsed in Krebs-Henseleit (KH) buffer, pH 7.4, containing 10 mM glucose, then opened longitudinally and mounted in modified Ussing chambers (29) with an exposed area of 1 cm². The KH buffer (4 ml) containing 1% BSA was added to each half of the chamber, and 100 pg of [⁵⁷Co]Cbl (25,000 cpm) were added to the chamber of either the mucosal or the serosal side of the ileum.
The temperature was maintained at 37°C with a water jacket, and a steady stream of 95% O2-5% CO2 was used to maintain the pH and circulate the buffer. The potential difference (PD) and short-circuit current were measured as previously described (22) using salt bridges of 2% agar in KH buffer in contact with Ag-AgCl electrodes, and resistance was calculated using Ohm's law. At 30, 60, and 120 min, buffer (0.5 ml) was removed from the mucosal and the serosal sides to determine TCII-bound [57Co]Cbl as described above, and the volume removed was replaced with fresh buffer.

Northern blot analysis of TCII mRNA. Total RNA was prepared from ileal segments using the guanidinium isothiocyanate-cesium chloride density gradient method (7). Thirty micrograms of total RNA were subjected to electrophoresis in a denaturing formaldehyde-1% agarose gel and then transferred to a nitrocellulose membrane and hybridized for 18 h to the 32P-labeled human TCII cDNA (21) in 50% formamide, 5× sodium chloride-sodium phosphate-EDTA (SSPE), 0.5% SDS, 10% dextran sulfate, and 100 µg/ml salmon sperm DNA. The membrane was then washed with 2× sodium chloride-sodium citrate (SSC)-0.1% SDS at room temperature for 1 h followed by 0.1× SSC-0.2% SDS at room temperature for 30 min. The final washing was carried out at 42°C in 0.1× SSC-0.1% SDS for 1 h.

In situ hybridization studies. The probe for in situ hybridization was selected from a region of the cDNA corresponding to a coding region in exon 2 that lacked any homology with transcobalamin I and I. The specific fragment was obtained by digesting the cDNA with Fok I and Hind III. A 94-bp fragment was purified and subcloned into the transcription vector, pGEM-3Zf(+) (Promega, Madison, WI). The plasmid was linearized, and radiolabeled sense and antisense transcripts were prepared using the MaxiScript RNA transcription kit (Ambion, Austin, TX) and α-35S-UTP (Amersham, Arlington Heights, IL). The radiolabeled probes were purified by electrophoresis in a 6% (19:1) polyacrylamide-8 M urea gel, and the specific band was eluted overnight at 37°C in 0.5 M ammonium acetate (pH 8.0)-1 mM EDTA. The eluted probes were concentrated by ethanol precipitation and dissolved in 100 mM dithiothreitol before use.

Segments (0.5 cm) of the distal ileum were obtained as described above and immediately fixed in 4% paraformaldehyde-buffered with 0.1 M sodium phosphate buffer, pH 7.4. The fixed tissue was sent to Novagen (Madison, WI) by overnight delivery for further processing. Sections (5–10 µm) of the ileum embedded in paraffin and mounted on Vectabond (Vector, Burlingame, CA)-coated slides were provided by Novagen.

For the in situ hybridization, the sections were deparaffinized by treating the slides with three changes of xylene, and the fixed tissue was hydrated through a graded series of ethanol (100, 95, 70, 50, and 30%). After washing the sections with 10 mM HEPES, pH 7.4, 0.15 M NaCl, the sections were treated for 5 min with 0.2 M HCl. After a RNase-free water wash, the sections were deproteinized with 1 µg proteinase K/ml water for 5 min at room temperature. After a second water wash, the slides were immersed in 0.1 M triethanolamine-HCl, pH 8.0, and acetic anhydride was slowly added to this solution while mixing to a final concentration of 0.25%. After 5 min the slides were again washed with the RNase-free water and the sections were incubated with 500 µl of the prehybridization solution (Novagen) for 2 h at 42°C in a humidified box. The prehybridization solution was replaced with 25 µl of hybridization solution containing 108 cpm of the radiolabeled RNA transcript per tissue section. The hybridization solution was covered with a sterile siliconized glass coverslip, and the edges were sealed with rubber cement.

Hybridization was carried out in the humidified box for at least 18 h at 42°C.

After hybridization, the glass coverslips were removed by soaking the slides for 5 min in 2× SSC prewarmed to 45°C. The slides were then washed for 30 min at 45°C with fresh 2× SSC-10 mM β-mercaptoethanol (ME), and the sections were treated for 30 min at 37°C with RNase A (20 µg/ml) in 0.5 M NaCl-10 mM β-ME. The washing was continued for 30 min at 45°C with 2× SSC, 50% formamide, and 10 mM β-ME and for 60 min at 45°C with 1× SSC, 14 mM β-ME, and 0.06% sodium pyrophosphate. The sections were dehydrated through graded ethanol and allowed to air dry at room temperature and then dipped in LM-1 hypercoat (Amersham) to coat the tissue section with photographic emulsion.

The coated slides were air dried and stored at 4°C in foil-wrapped plastic boxes containing desiccant, were developed after 1–11 days with D-19 (Kodak, Rochester, NY) for 5 min at 15°C, followed by a 30-s wash in deionized water, and were fixed for 10 min with Kodak fixer. The fixed slides were rinsed with tap water, dried at room temperature, and stained with hematoxylin for 2 min.

RESULTS

TCII secretion by ileal segments. Freshly obtained ileal segments contained trace amounts of TCII in the 100,000 g supernatant fraction of the homogenized scraped mucosa. When these segments were cultured at 37°C as outlined in METHODS and [57Co]Cbl was added to the medium, a time-dependent increase in QUSO-precipitable radioactivity was observed over the 120-min period (Fig. 1). A similar increase of TCII in the culture medium occurred as previously reported (28) when IF-[57Co]Cbl was instilled in the ileum in vivo for 2–3 h before the ileal segments were cultured. This increase in TCII-[57Co]Cbl did not occur when the segments were incubated at 4°C. When tissue was fully submerged in the medium, or when unopened ileal segments were submerged in the medium, TCII was not detected, indicating that viability (i.e., oxygenation) of the tissue was required for the synthesis of TCII.

![Fig. 1. Accumulation of transcobalamin (TCII) in medium of cultured segments of distal ileum. Values are expressed as pg of TCII protein, derived by multiplying bound [57Co]cobalamin (Cbl) by 32 (molar ratio of molecular weight of TCII to molecular weight of Cbl).

□, Incubation at 37°C; ○, incubation at 4°C. Each point is mean ± SD of 3 separate experiments.](http://ajpgi.physiology.org/DownloadedFrom/10.220.32.247/March31,2017)
TRANSCOBALAMIN II AND COBALAMIN ABSORPTION

No apo-TCII was detected when Cbl was omitted from the culture medium or when Cbl bound to IF or R-binder from saliva was added to the medium. TCII was not detected in the absence of Cbl, even when carrier protein (10% BSA) and protease inhibitors (10 U/ml Trasylol and 100 µM phenylmethylsulfonyl fluoride) were added to the culture.

The directional transport of TCII was determined by adding [57Co]Cbl to the mucosal or serosal side of a segment of ileum placed in an Ussing chamber. The PD recorded at time 0 was −9.3 ± 2.4 mV and resistance was 99 ± 7 Ω/cm². These values were unchanged at 30 min but did change sufficiently at 60 min to indicate that tissue viability may have been compromised. Hence only the results of the 30-min time point are shown (Fig. 2). Approximately 68% of the free [57Co]Cbl added to the serosal side of the ileum was recovered as TCII-[57Co]Cbl, with less than 1% appearing on the mucosal side. However, only 32% of the free [57Co]Cbl added to the mucosal side of the ileum was recovered in the buffer as TCII-[57Co]Cbl on that side, and ~7% of the [57Co]Cbl added to the mucosal side appeared on the serosal side bound to TCII. These findings indicate that the principal direction of TCII transport following its synthesis and secretion is toward the serosal side, which, in vivo, would be into the microcirculation in the villus. The finding of TCII-[57Co]Cbl on the mucosal side of the ileum, however, indicates that apo-TCII passes to that side through the interstitial spaces between the enterocytes. Studies by Anderson and co-workers (1) have established that serum proteins with a molecular mass of <100 kDa can pass into the intestinal succus through the intercellular space between the enterocytes.

Figure 3 shows the Northern blot of total RNA extracted from the guinea pig small intestinal mucosa and probed with the radiolabeled human TCII cDNA. A 1.9-kb transcript corresponding to the TCII mRNA (21) was detected in the proximal jejunum, a region of the small intestine that does not express the IF receptor, as well as in the middle and the distal ileum, where IF-Cbl is absorbed. The TCII mRNA in the ileal tissue did not increase when IF-Cbl was incubated in the lumen in vivo for 2–3 h before the RNA was prepared for Northern blotting.

In situ identification of TCII mRNA in the terminal ileum. In situ hybridization localized the transcript predominantly to the crypt region and the lower third of the villus (Fig. 4A, arrows). Moreover, dense areas of grains were also observed at the junctional region of the base of the columnar mucosal cell and the apposing central core structure of the villus (Fig. 4B, arrow). Significantly, there were only a few grains in the columnar epithelial cells that line the luminal surface of the villus (Fig. 4, A and B, arrowheads). The negative control obtained by hybridization with the 35S-labeled sense transcript in Fig. 3, C and D, shows no radiographic grains in the corresponding regions of the mucosa.

DISCUSSION

The transfer of Cbl from the intestinal lumen to the portal blood requires a complex sequence of interactions of IF-Cbl in the succus entericus with a receptor for this complex on the brush border of the mucosal cells, followed by internalization of the complex via receptor-mediated endocytosis (16, 27). Within the endosome, Cbl is released from IF (8, 12) for transfer to the portal blood and systemic circulation complexed with TCII (28). The observation that congenital deficiency of TCII is associated with intestinal malabsorption of Cbl (4, 13) has suggested a role for this protein in Cbl absorption through the ileal mucosa. However, the source of TCII in the ileum and the point in the absorptive process at which Cbl binds to this transport protein have not been clear.

The initial experimental evidence that TCII participates in Cbl absorption was the serendipitous observation that TCII-Cbl appeared in the medium when a segment of guinea pig ileum was cultured following in vivo incubation of IF-[57Co]Cbl in the lumen of the intestine (28). Other studies also supported this observation. Katz and O’Brien (17) observed that [57Co]Cbl appeared in the superior mesenteric venous drainage bound to a protein similar in size to TCII when rats...
were fed $[^{57}\text{Co}]\text{Cbl}$ and the intestine was perfused ex vivo. Robertson and Gallagher (27) identified TCII-bound $[^{57}\text{Co}]\text{Cbl}$ in the solubilized homogenate of the ileal mucosa of mice 2–4 h following an oral dose of $[^{57}\text{Co}]\text{Cbl}$. Kapadia and co-workers (16) observed TCII-$[^{57}\text{Co}]\text{Cbl}$ in isolated enterocytes 4–5 h after human IF-bound $[^{57}\text{Co}]\text{Cbl}$ was instilled into a loop of distal ileum of guinea pigs. However, 2 h after the instillation of the IF-$[^{57}\text{Co}]\text{Cbl}$ into the loop, most of the $[^{57}\text{Co}]\text{Cbl}$ was either free or still bound to IF. Studies by Chanarin et al. (6) in humans showed that when circulating TCII is saturated with $[^{57}\text{Co}]\text{Cbl}$, orally administered $[^{58}\text{Co}]\text{Cbl}$ appears in the blood bound to TCII. This could only occur if apo-TCII is available in the ileum at the site of Cbl absorption and in close apposition to the basolateral surface of the epithelial cells where free Cbl is released (8, 16).

Our initial observation that TCII did not appear in the culture medium when Cbl was omitted from the experimental system suggested that the vitamin is needed for the expression of TCII, especially since no TCII was detected even when protease inhibitors and carrier protein were added to the medium. However, this notion could not be validated because apo-TCII added to the culture medium could not be recovered, indicating that the protein was degraded. This phenomenon may be an artifact of the in vitro culture conditions whereby the resected tissue releases proteases because of cellular damage. Indeed, meprin, a metalloprotease, has been identified in intestinal mucosa and is not inactivated by the conventional serine protease inhibitors (2). Thus it is likely that Cbl binding to the nascent apo-TCII protects it from proteolysis.

The identification of TCII mRNA in the jejunum, as well as the ileum, is consistent with the constitutive expression of TCII by the vascular endothelium, an integral component of the microcirculation of the villi of the entire small intestine. It is, therefore, reasonable to speculate that if the mucosa of the proximal small intestine could express the receptor for the IF-Cbl complex, transfer of Cbl from the epithelium to TCII would occur there as it does in the distal ileum.

The predominant distribution of the autoradiographic grains to the crypts, the lower third, and central core of the villus, with substantially fewer grains in the columnar cells, is consistent with the localization of the TCII transcript to the endothelium of the microcirculation within the villus and is consonant with previous studies showing that endothelial cells in culture and in situ synthesize TCII (23). In the absence of TCII, Cbl released from IF within the endosome is likely to be lost with the shedding mucosa, and this is the likely reason for the Cbl malabsorption observed in congenital TCII deficiency (4).

The alternative notion that TCII is synthesized in the epithelial cells of the ileal mucosa stems from three previous observations and merits comment. In the first study, combining in vivo incubation of human IF-$[^{57}\text{Co}]\text{Cbl}$ in an ileal loop of a guinea pig followed by culturing the ileal segment for 3 h, the appearance of TCII-$[^{57}\text{Co}]\text{Cbl}$ both in the mucosal homogenate and medium led to the conclusion that the enterocyte was the source for the TCII (28). This determination was also based on positive indirect immunofluorescent imaging for TCII in the mucosa of the cultured segment. In retrospect, such immunofluorescence of the entire mucosa might also be ascribed to TCII-Cbl in the interstitial fluid surrounding the epithelial layer of the mucosa. The TCII-Cbl complex that forms as free Cbl exits the abluminal side of the mucosa would then surround the enterocytes as well as pass into intestinal fluid. This would also account for the TCII-$[^{57}\text{Co}]\text{Cbl}$ on the mucosal side of the Ussing chamber when free $[^{57}\text{Co}]\text{Cbl}$ was placed on the mucosal side in that experiment.

A second study that appeared to indicate the synthesis of TCII by the intestinal enterocyte included the finding that a solubilized homogenate, prepared from a segment of rat intestine after oral administration of IF-$[^{57}\text{Co}]\text{Cbl}$, contained $[^{57}\text{Co}]\text{Cbl}$ bound to immunoprecipitable TCII, and this transfer of IF-bound $[^{57}\text{Co}]\text{Cbl}$...
to TCII was inhibited by the coadministration of chloroquine (27). However, the finding of TCII-[57Co]Cbl in a solubilized crude homogenate does not provide sufficient discrimination between the tissue components of the ileum to conclude that the enterocyte is the source of the TCII. Kapadia et al. (16) failed to detect TCII expression in isolated guinea pig ileal enterocytes after the internalization of IF-[57Co]Cbl by the cells. However, when IF-[57Co]Cbl was instilled into an ileal loop and the enterocyte prepared subsequently, TCII-[57Co]Cbl was identified in the isolated enterocyte 2–4.5 h later. The internalization of the TCII-[57Co]Cbl could occur via the receptor for this complex on the basolateral surface of the enterocyte by a process similar to that observed for the uptake of transferrin-bound iron via the transferrin receptor in the proximal small intestine (19).

Finally, the observation that Caco-2 cells, a cell line derived from a human colon carcinoma, may, under certain culture conditions, express both the IF-Cbl receptor and TCII (25) appeared to support the conclusion that normal ileal enterocytes also synthesize TCII (26). Although such an analogy may be valid in some instances, neoplastic cells may express a variety of proteins, or other products of cell metabolism, that the normal counterpart cell does not. This is an especially important consideration for TCII, because blood levels of this protein are elevated in a number of neoplastic disorders (5, 18). Moreover, Dan and Cutler (8) also studied transcytosis of Cbl in Caco-2 cells and did not find TCII-Cbl in the basolateral medium following uptake of IF-Cbl, and most of the intracellular Cbl was free. Thus there is substantial evidence (16, 17, 27) that the enterocyte is not a primary source of TCII in the villi. Rather, the findings in this study support our hypothesis that TCII is synthesized in the vascular villi. Rather, the findings in this study support our hypothesis that TCII is synthesized in the vascular villi. Rather, the findings in this study support our hypothesis that TCII is synthesized in the vascular villi. Rather, the findings in this study support our hypothesis that TCII is synthesized in the vascular villi.

REFERENCES


