

Immunocytochemical Localization of the Intrinsic Factor-Cobalamin Receptor in Dog-Ileum: Distribution of Intracellular Receptor during Cell Maturation

JOEL S. LEVINE, ROBERT H. ALLEN, DAVID H. ALPERS, and BELLUR SEETHARAM
Department of Medicine, Divisions of Gastroenterology and Hematology, University of Colorado School of Medicine, Denver, Colorado 80262; and Division of Gastroenterology, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT Absorption of cobalamin is facilitated by the binding of the intrinsic factor-cobalamin complex (IF-cbl) to specific receptors in the ileum. The physical and biochemical characteristics of this ligand-receptor binding reaction have been extensively studied, but little is known about the cellular mechanisms or receptor synthesis, intracellular transport, and expression on the microvillus surface membrane. We attempted to delineate these mechanisms by using ultrastructural immunocytochemistry to localize the IF-cbl receptor in the crypt, mid-villus, and villus tip regions of mucosal biopsies obtained from the ileum of anesthetized dogs. Prior to initiating the ileal localization studies, the antisera to purified canine IF-cbl receptor that was employed in our studies was shown to have specificity for site (e.g., ileal enterocytes vs. other cells within the gastrointestinal tract) and immunohistochemical specificity. Receptor synthesis in endoplasmic reticulum begins in crypt enterocytes, but continues in cells throughout the villus. In the mid-villus region synthesized receptor translocates vectorially to the microvillus surface associated with membranous vesicles and then inserts into the microvillus pit. Receptor remains fixed to the microvillus pit and does not distribute uniformly over the brush border membrane. All villus tip enterocytes contained IF-cbl receptor in microvillus pits, vesicles, and endoplasmic reticulum, but in addition extensive perinuclear membrane staining was evident as well as re-internalized receptor associated with multivesicular bodies. Basolateral membranes contained no receptor at any level of the villus. These observations suggest that the IF-cbl receptor (a) translocates to the apical cell surface at the mid-villus region by transport in vesicles, (b) directly inserts into and then remains fixed in microvillus pits, (c) is elaborated on the luminal surface most extensively in villus tip cells, and (d) although reinternalized, does not move IF and/or cbl to the basolateral cell surface.

In adult mammals the absorption of physiologic quantities of dietary cobalamin (vitamin B₁₂, cbl¹) requires that a complex of cbl and intrinsic factor (IF) bind to specific receptors in the ileum. The binding of the IF-cbl complex to the receptor does not require energy, but does need Ca⁺⁺ and a neutral pH (1–3). We have recently demonstrated (4) that IF-cbl only

binds to the pits of microvilli in the upper one half of villi in guinea pigs. This provided indirect evidence that IF-cbl receptor expression on the luminal surface membrane occurred late in cell maturation, and that once inserted into this membrane the receptor was not distributed over the entire microvillus surface.

Using immunoaffinity chromatography, Seetharam and co-workers have purified (65,000-fold) and characterized the IF-cbl receptor from the canine ileum. The receptor is a large

¹ *Abbreviations used in this paper:* IF-cbl, intrinsic factor-cobalamin complex; RER, rough endoplasmic reticulum; TC, transcobalamin.

protein ($200\text{--}222 \times 10^3$ daltons) that specifically binds IF-cbl but not free cbl or IF (5). Reconstituting the receptor using cationic liposomes revealed that most of the receptor is located on the luminal side of the membrane, and the IF-cbl binding sites are exposed to the lumen (6). More recent studies (7) confirm that the receptor is an integral membrane protein, over 80% of which extends beyond the lipid bilayer. The cellular mechanisms of receptor expression, and the anatomical location of this protein are unknown.

Therefore, using antisera generated in rabbits to this highly-purified receptor protein (5), we undertook an ultrastructural immunoperoxidase study to define the cellular distribution of IF-cbl receptor in canine intestine during enterocyte maturation from the crypt to the villus tip.

MATERIALS AND METHODS

Sources of Proteins and Antisera

Homogeneously purified hog IF (3), human transcobalamin II (TC II) (8), and highly purified canine IF-cbl receptor (5) were obtained as described previously.

Characteristics of the rabbit anti-dog IF-cbl receptor antisera, described in detail elsewhere (5), are that (a) it precipitates >80% of ^{125}I -labeled receptor; (b) it inhibits the binding of the IF- ^{57}Co cbl complex to the purified receptor or to isolated brush borders; and (c) when used in a 30-fold excess, low affinity anti-IF antibody activity is identified. The latter property is probably due to a trace contamination with hog IF in the purified receptor used as an immunogen. The source of affinity purified Fab' fragments to sheep anti-rabbit gamma globulin, and their conjugation to horseradish peroxidase have been described previously (9). All antisera were diluted in phosphate buffered saline (PBS)-10% sucrose-1% BSA.

Immunocytochemical Protocol

All tissue examined in this study was processed using an indirect immunocytochemical protocol described in detail previously (10). In this study all tissue was fixed for 4 h at room temperature. Serial $6\text{-}\mu\text{m}$ sections were cut on a cryostat after the fixed intestine had been washed in PBS containing sucrose. The first antibody placed on the sections was either the anti-dog IF-cbl receptor antiserum, an adsorbed control antiserum (see below) or normal rabbit serum. The first antibody was left on the sections for 20 h. A 1:45 dilution of the sheep-anti-rabbit Fab'-horseradish peroxidase was placed on for 3 h. After staining, the $6\text{-}\mu\text{m}$ tissue sections were embedded and coded prior to ultramicrotomy. Silver-gold sections, obtained at $1\text{-}\mu\text{m}$ levels through the tissue, were viewed on a Philips 201 electron microscopy at 60 kV. Each coded block was evaluated as positive or negative for IF-Cbl receptor and whether ultrastructure was preserved. If positive for IF-Cbl receptor, the intracellular location of any immunogenicity was documented and recorded for each cell visualized (see below). The blind was broken only after all tissue from an individual dog experiment had been evaluated. In our hands counterstaining with heavy metals has been found to cause false positive immunoreactivity, and thus was not routinely employed, except when attempting to define "coating" of microvillus pits (Fig. 6).

Experimental Protocols

PRELIMINARY STUDIES: After examining several fixatives (paraformaldehyde-picric acid, periodate-lysine-paraformaldehyde, and glutaraldehyde-paraformaldehyde), it was found that picric acid-paraformaldehyde (11) provided the optimal morphologic preservation of mucosa while maintaining immunogenicity. Sequential studies revealed that the optimal working dilution of the rabbit anti-dog IF-cbl receptor antiserum was 1:20,000, and of the rabbit anti-dog IF-cbl receptor Fab' 1:40.

TISSUE SPECIFICITY: Mucosal biopsies (0.5×0.5 cm) were obtained from the stomach (fundus and pylorus), duodenum, jejunum, ileum, and colon of four healthy anesthetized adult dogs. The tissue was fixed in paraformaldehyde-picric acid and processed for immunocytochemistry (as above).

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF ANTIRECEPTOR ANTISERA: To determine immunocytochemical specificity the anti-receptor antisera was (a) replaced with normal rabbit serum (NRS); (b) adsorbed with highly purified IF-cbl receptor; (c) adsorbed with IF, IF-cbl, and TC II; and (d) adsorbed with IF-cbl receptor bound to IF-cbl complex.

DISTRIBUTION OF IF-CBL RECEPTOR DURING VILLUS MATURATION: Mucosal biopsies from the ileum (10-cm proximal to ileocecal valve) of eight anesthetized adult dogs (2-4 years of age) were processed for immunocytochemistry. Prior to ultramicrotomy the best oriented villi were selected, and portions of the crypt, mid-villus, and villus tip areas were blocked off (Fig. 1) and sectioned. These areas were obtained from both positive and histochemical control sections.

Ultrastructural immunocytochemistry, as undertaken in this study, cannot quantify the amount of IF-cbl receptor in specific subcellular compartments. However, after viewing several biopsies it became obvious that there were dramatic differences in the cellular distribution of IF-Cbl receptor at the three levels of mucosa examined. In an attempt to express these differences, the location of receptor within specific cellular compartments was recorded for all intact enterocytes present at the mid-level (third grid) of each $6\text{-}\mu\text{m}$ tissue section. If any portion of a specific organelle (e.g., part of the perinuclear membrane) was stained, it was recorded as positive. These counts were expressed as the percentage of cells visualized, to the nearest 10%, that contained a specifically stained organelle. Our definitions of subcellular compartments are: (a) rough endoplasmic reticulum (RER): tubular structures that had a granular appearance. Counterstaining with uranyl acetate and lead citrate identifies polyribosomes on these structures when they are unstained (9); (b) golgi apparatus: smooth, tubular, stacked membranous structures; (c) Perinuclear membrane: the percentage was derived as percent of nuclei with receptor staining (since some cells had no nuclei at that level); (d) multivesicular bodies: round-ovoid, smooth-membraned organelles containing small membranous vesicular structures; (e) microvilli: those portions of the surface membrane that project into the lumen. Microvillus pits: that portion of the surface membrane between the base of adjacent microvilli. Selected grids were counterstained with uranyl acetate and lead citrate to see whether "coated" pits could be identified; (f) basolateral membrane: that portion of the surface membrane beneath the cellular tight junctions; (g) Membranous vesicles: tubular or round, membranous structures.

RESULTS

Tissue Specificity

Immunoreactive IF-cbl receptor was only found in enterocytes in ileal mucosa. Stomach (fundus, pylorus), duodenum, jejunum, and colon mucosa did not stain when plated with the antireceptor antisera (Table I).

Immunohistochemical Characterization of the Antireceptor Antibody

Replacing the antireceptor antisera with normal rabbit serum or anti-IF-cbl receptor adsorbed with purified receptor completely abolished staining (Fig. 2). Immunoreactivity was also abolished when the antisera was adsorbed with receptor

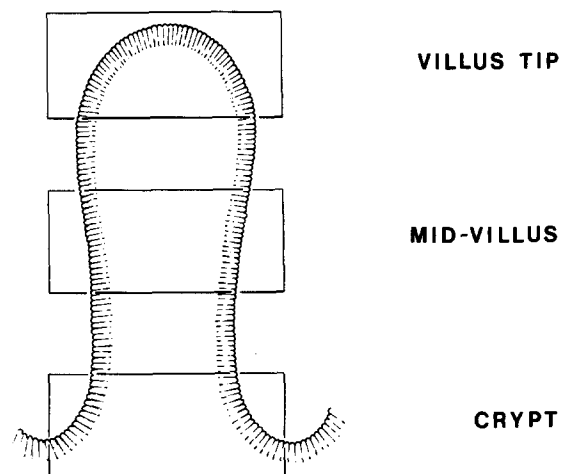


FIGURE 1 Illustration of which portions of the intact villus were blocked off for ultramicrotomy to obtain sections of the crypt, mid-villus, and villus tip.

bound to the IF-cbl complex. However, preincubation of the antireceptor antisera with IF, IF-cbl, or TC II did not diminish staining of the receptor within enterocytes (Table I).

Distribution of IF-cbl Receptor during Enterocyte Maturation

There was no difference in intracellular location of receptor between (a) the grids obtained through the entire thickness of the original sections, (b) anti-IF-cbl-receptor used as a dilution of whole serum or an Fab' fragment, (c) multiple sections of biopsies from the same dog, or (d) between the same level of the villus in different dogs. All receptor was confined to ileal enterocytes, with none in goblet or enterochromaffin cells.

CRYPT: IF-cbl receptor was identified in only 1 in 30 crypt enterocytes and was present in RER (Fig. 3a). There was no receptor in other cellular organelles or on the microvillus membrane (Fig. 3b).

TABLE I
Tissue and Immunohistochemical Specificity of Anti-intrinsic Factor-Cobalamin-Receptor (α IF-Cbl-R) Antisera

Immuno-reactive IF-Cbl-R	Tissue	First antibody
+	- Ileum	- α IF-Cbl-R
		- α IF-Cbl-R + IF-Cbl
		- α IF-Cbl-R + TC II
		- α IF-Cbl-R + IF
-	- Ileum	- NRS
	- Ileum	- α IF-Cbl-R + IF-Cbl-R
	- Stomach	
	- Duodenum	- α IF-Cbl-R
	- Jejunum	
	- Colon	

+, Normal receptor staining pattern; -, absence of any immunoreactive IF-Cbl-R

MID-VILLUS: 30-50% of the enterocytes at the mid-villus area contained receptor. In all of these cells receptor was present in RER, but in addition was also associated with membranous tubular vesicles scattered between RER and the microvillus surface. At the luminal surface focal microvillus pits stained for the receptor (Fig. 4a). Many tubular vesicles containing receptor appeared to be in direct continuity with microvillus pits (Fig. 4b). At this level rare Golgi apparatus, but not perinuclear membrane, basolateral membrane, or multivesicular bodies contained IF-cbl receptor.

VILLUS TIP: The degree of IF-cbl receptor staining was greatest in villus tip enterocytes. Essentially all (>95%) enterocytes at this level contained receptor, with extensive demonstration of receptor in microvillus pits (Figs. 5-7). Receptor at the luminal surface was only present in microvillus pits, and not present diffusely over the brush border membrane. When selected sections were counterstained with uranyl acetate and lead citrate, the stained and unstained pits did not appear to demonstrate a "coat" (Fig. 6). In addition to the tubular vesicles and RER (Fig. 5) seen at the mid-villus region, IF-Cbl receptor was now associated with perinuclear membrane (Fig. 5), Golgi apparatus, and multivesicular bodies (Fig. 7). Receptor was never associated with basolateral membrane, mitochondria, or cytosol. The distributional changes in intracellular IF-cbl receptor that occurred during cell maturation are summarized in Table II. It is clear that the smooth tubular vesicles were seen in nearly every cell, but the multivesicular bodies in only 10%.

DISCUSSION

After demonstrating the tissue and immunohistochemical specificity of the anti-IF-cbl receptor antisera, we were able to identify consistent changes in the intracellular distribution of the IF-cbl receptor during the maturation of canine ileal enterocytes. By localizing intracellular IF-cbl receptor in ileal enterocytes from the crypt to the villus tip, we have been able

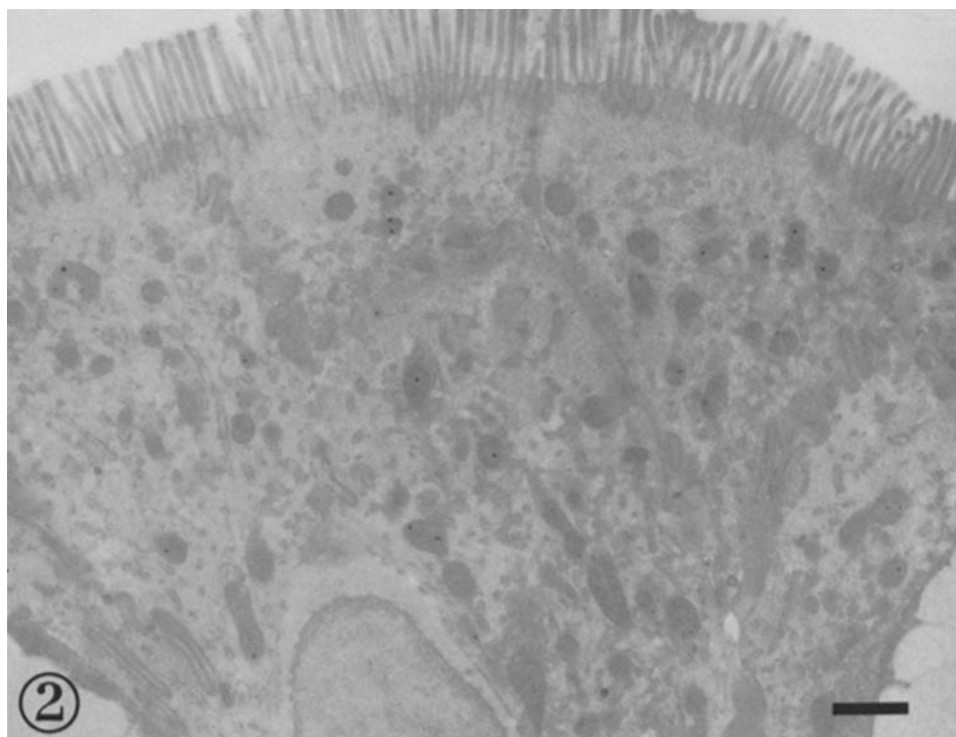


FIGURE 2 Electron micrograph of the villus tip of canine ileum reacted with antidog intrinsic factor-cobalamin (anti-IF-cbl) receptor that has been adsorbed with purified receptor. There is no immunoreactive receptor present. Bar, 1 μ m. \times 9,900.

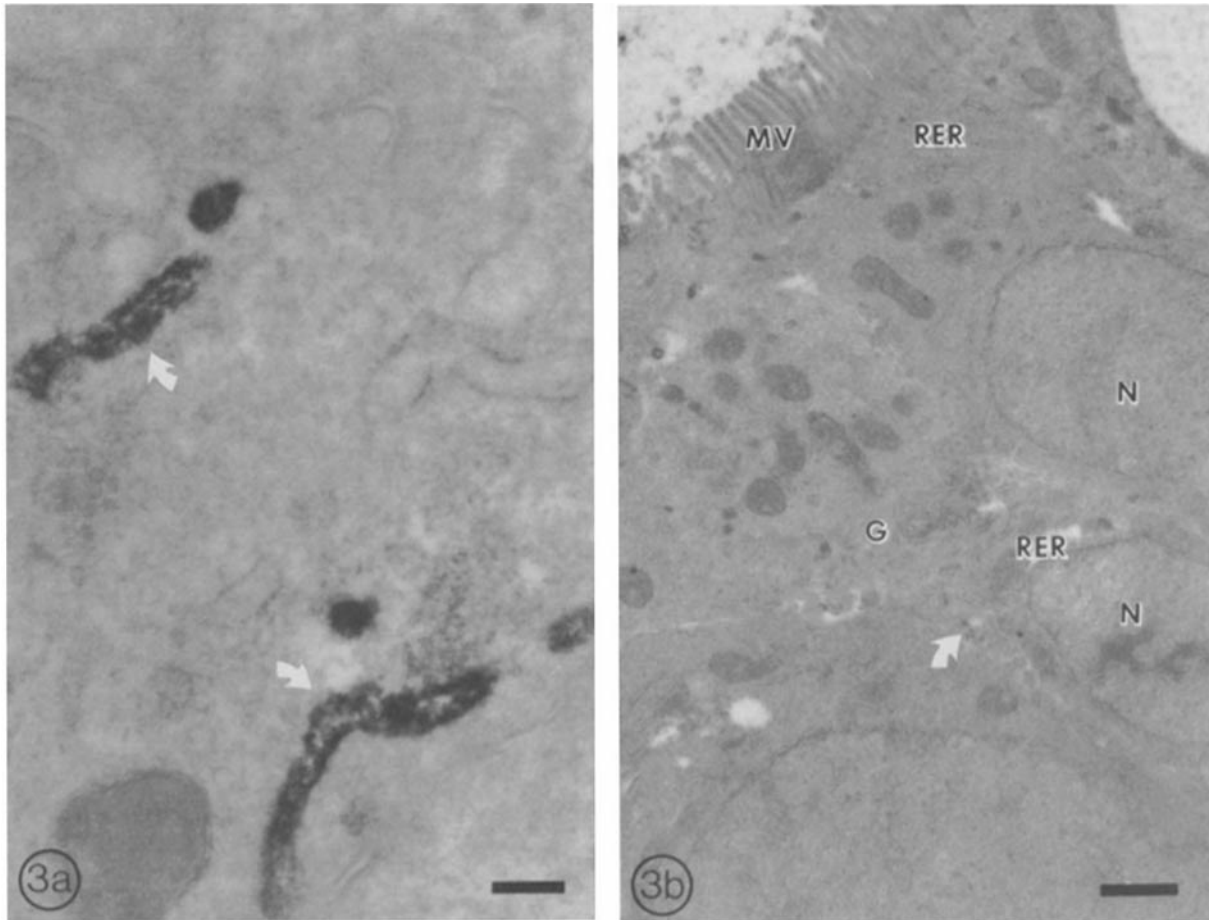


FIGURE 3 Electron micrographs of the crypt from dog ileum that has been reacted with anti-IF-cbl receptor. (a) IF-cbl receptor is present on focal rough endoplasmic reticulum in 1 out of 30 crypt cells visualized (curved white arrows). (b) At lower power it is apparent that the majority of rough endoplasmic reticulum (RER), all of the Golgi apparatus (G), membrane surrounding the nucleus (N), and the microvillus surface membrane (MV) contain no immunoreactive IF-cbl receptor in the crypt. Bars, 0.2 μm (a); 1 μm (b). $\times 50,000$ (a); $\times 9,900$ (b).

to place the visualized events within a time frame. This time frame (newly formed cells in the crypt, oldest cells at the villus tip) then enabled the direction of intracellular movement of localized receptor to be inferred. This was only possible because the receptor's expression at its biologically active site (the brush border) occurred sequentially along the villus, with most of the microvillus receptor present at the villus tip. If the receptor had been more uniformly distributed over the villus, as has been shown for sucrase (12), then it is unlikely that differences would have been noted, or that direction of movement could have been inferred.

Translocation of the receptor from endoplasmic reticulum synthetic sites to the microvillus surface takes place in the mid-villus region, and appears to depend on vesicular transport. That receptor, at this level, is associated with RER and tubular membranous structures in the same cells that have a paucity of microvillus staining (Fig. 4a), and that many of these vesicles appear to be in direct continuity with stained microvillus pits (Fig. 4b) strongly support this formulation. The importance of membrane bound vesicular transport in delivering substances to the cell surface (9, 13), has been previously demonstrated. The absence of receptor on the basolateral surface membrane at any level of the villus suggests that this membrane-associated protein moves to the apical surface membrane directly, as does oligoaminopeptidase in

the enterocyte (14). In contrast, Hauri et al. (15), using cell fractionation of intestinal mucosal cells, have suggested that another brush border enzyme, sucrase, is initially inserted in the basolateral membrane, and then moves across the tight junction to be expressed at the brush border. Although it is possible that the absence of receptor staining on basolateral membrane in our study reflects an inaccessibility of our antibody to this membrane site, the direct contact between vesicles and microvillus pits and the clarity of our intracellular membrane staining make this possibility unlikely. Since it has been shown (14, 16) that the basolateral cell membranes may be contaminated by Golgi membrane in cell fractionation studies, the concept of movement across tight junctions of brush border membrane proteins remains to be clearly documented.

We have demonstrated that the IF-cbl receptor is elaborated on the luminal surface membrane on canine ileal enterocytes in the upper one-half of the villus. It appears that once the receptor is inserted into the microvillus pit it remains relatively fixed in this position, whereas other essential brush border components that are also membrane-associated (i.e., enzymes) have a more uniform distribution (17). Since the IF-cbl complex has also been shown to bind solely to microvillus pits in guinea pig ileum (4), it is unlikely that a simple technical inability to demonstrate the intracellular receptor

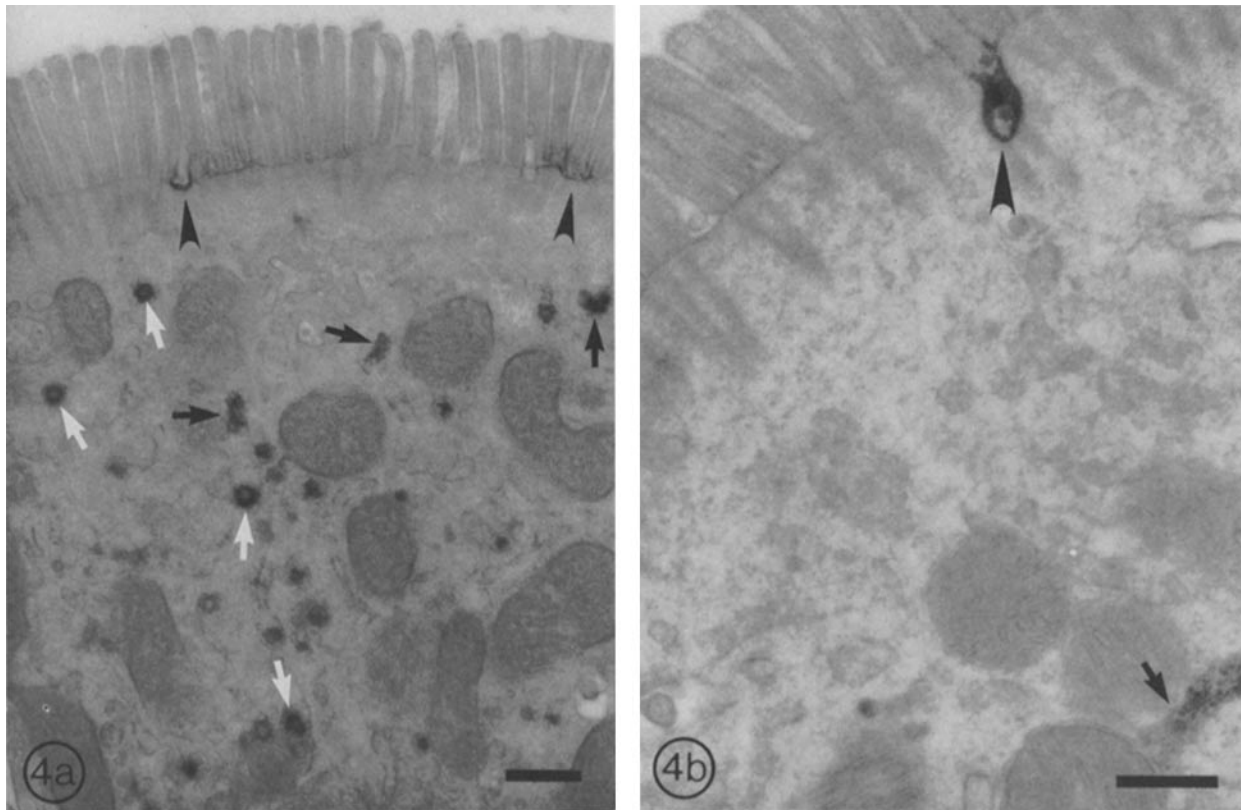


FIGURE 4 Electron micrographs of the mid-villus region from dog ileum that has been reacted with anti-IF-cbl receptor. (a) Immunoreactive receptor is associated with a large number of membranous vesicular structures below the microvillus surface. Some of the vesicles have a tubular shape (black arrows), where as others are circular (white arrows). This probably reflects the plane of sectioning. Additionally, receptor is present on focal microvillus pits at this level of the villus (black arrowheads). (b) The black arrowhead is pointing out what appears to be a receptor containing vesicle in direct continuity with a microvillus pit. Bars, 0.4 μm (a); 0.3 μm (b). $\times 25,500$ (a); $\times 42,500$ (b).

higher up on the microvillus can be implicated. Alternatively, the presence of the IF-cbl receptor in the pits might reflect an aggregation of receptor due to the presence of IF-cbl in the lumen. When receptors have been identified by ligand localization in isolated cell systems, such as low density lipoprotein (18), $\alpha 2$ -macroglobulin (19), and epidermal growth factor (20), they appear to insert randomly in the surface membrane and then aggregate within endocytotic pits on being placed in contact with the ligand. Thus, if IF-cbl were excluded from the lumen of the gut, it is possible that cell surface receptor location might become more diffuse, and receptor number increase.

Fixation of the receptor in the microvillus pit would readily permit endocytosis of the IF-cbl complex bound to the receptor as has been demonstrated for low density lipoprotein (18) and TC II (21) in fibroblasts, and asialoglycoproteins (22), including TC III (23) in the liver. Indeed, a number of investigators have suggested that receptor-mediated endocytosis of the IF-cbl complex is the initial cellular event in moving cbl from the lumen to the portal circulation (24, 25). Though we have shown some evidence of internalization of the surface receptor (see below), in our previous study in guinea pigs we did not localize IF within endocytotic vesicles (4). It is possible that IF immunoreactivity was lost, or IF could have become soluble, immediately after endocytosis. An alternative explanation might be that the IF-cbl complex is processed in or near the microvillus pit, that Cbl is dissociated in this area, and that IF then becomes available at the

luminal surface to bind more cbl as has been suggested by the studies of Hines (26). Our inability to demonstrate "coating" of the microvillus pits must be interpreted with caution (27). The immunoperoxidase method may obscure the demonstration of coating, and certainly the stained organelles have granular peroxidase on their surface. Whether clathrin actually penetrates the terminal web and binds to microvillus pits could only be defined by specifically localizing clathrin.

The presence of the IF-cbl receptor in multivesicular bodies presents strong morphological evidence that some of the receptor in the microvillus pits is internalized in villus tip enterocytes. In elegant studies of the tubular epithelium of the rat vas deferens (28), Friend and Farquhar were able to demonstrate that a soluble marker (horseradish peroxidase) placed in the lumen was first internalized in small coated vesicles and then accumulated in multivesicular bodies. The same sequence of events has been shown in the liver (13) and the intestine (29), and has led to the concept that the multivesicular body plays a role in the internalization and processing of surface membrane. What role, if any, the multivesicular body might play in receptor mediated endocytosis is unclear. Indeed, the inability to localize IF within multivesicular bodies in our previous study (4), the infrequency ($\sim 10\%$) of receptor in multivesicular bodies when $>95\%$ of villus tip enterocytes have receptor in microvillus pits, and the absence of stained coated pits could be explained if the IF-cbl complex, when bound to the microvillus receptor, prevents receptor internalization, rather than initiating endocytosis as does low

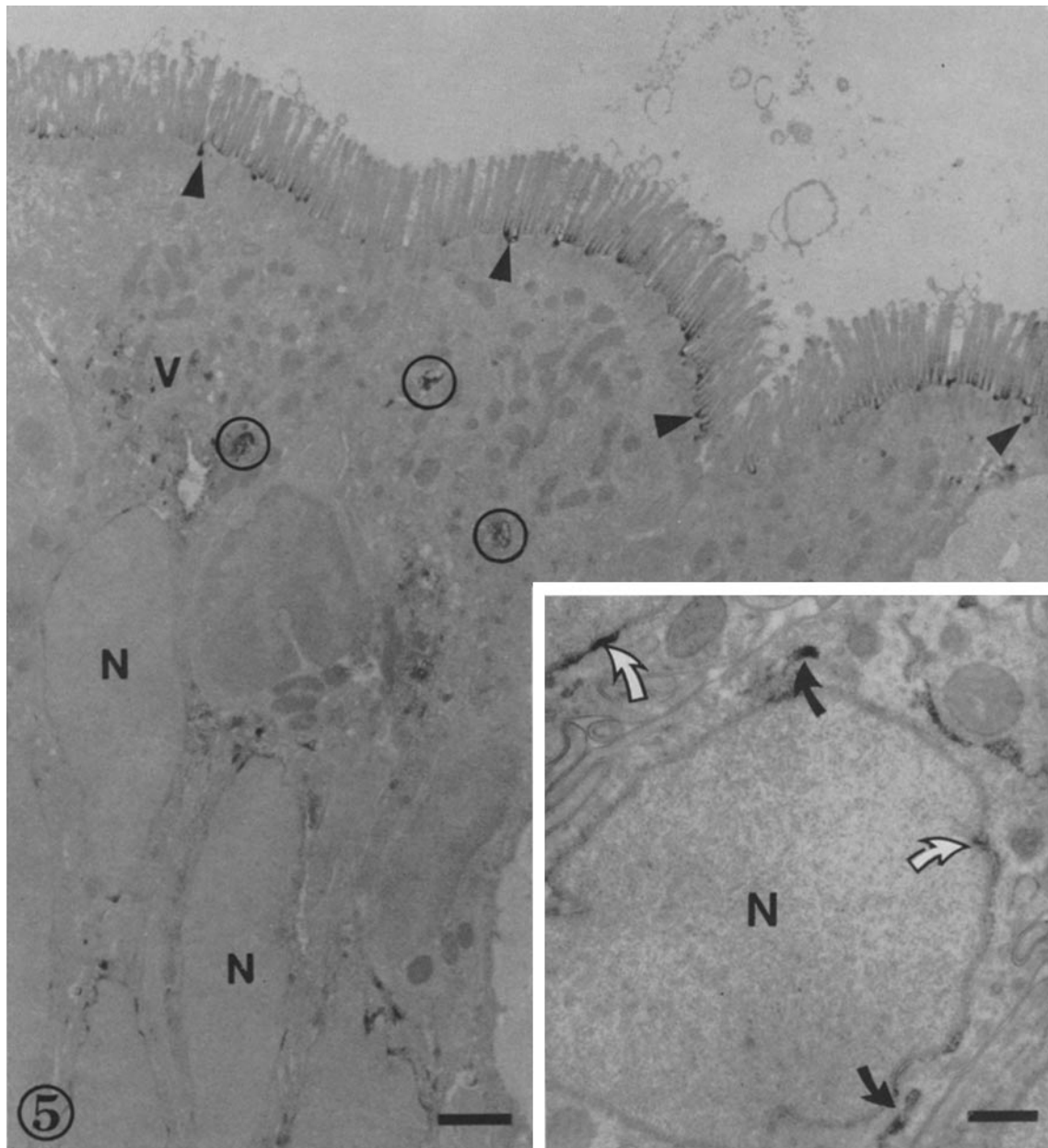


FIGURE 5 Electron micrographs of the villus tip region from dog ileum that has been reacted with anti-IF-cbl receptor. At this level immunoreactive receptor is present in the majority of microvillus pits (black arrowheads), in vesicles (V) between the nucleus (N) and the surface, and rough endoplasmic reticulum (curved black arrows, *inset*). In addition, villus tip enterocytes also demonstrate receptor in multivesicular bodies (circled in black), and in the membranous envelopes surrounding nuclei (N) (shown at higher power in the inset, curved white arrows). Receptor is not seen in the basolateral membrane. Bars, 1 μ m (a), 0.5 μ m (b). \times 9,800; \times 20,000 (*inset*).

density lipoprotein (18). Whether the IF-cbl receptor that is internalized in multivesicular bodies is digested within a lysosomal compartment or recycled back to the microvillus pit, cannot be determined from our study. The prominent staining of perinuclear membrane, only seen in villus tip enterocytes, raises the possibility that this might be one site of internalized receptor; or, as has been suggested by others (30), may simply reflect synthesis. However, the absence of receptor on the basolateral surface membrane provides strong evidence that the receptor is not involved in the shuttling of cbl (with or without IF) to the portal circulation.

In contrast to other endocytosed ligands, transferrin and IF are not degraded. After removal of iron, apotransferrin is

released into the medium (31, 30). It is interesting that transferrin is taken up into an acidic compartment of the cell which is not the lysosome (31, 32). Perhaps IF undergoes a similar fate.

In conclusion, using an immunohistochemically specific antisera raised in rabbits against homogeneously purified IF-cbl receptor, we have been able to demonstrate the intracellular distribution of the receptor in the canine ileum *in situ*. This distribution varies as the enterocytes mature, and as the receptor (a) is synthesized, (b) is translocated vectorially to the microvillus surface by vesicular transport, (c) remains fixed within the microvillus pit, (d) is internalized within multivesicular bodies, and (e) is not found on basolateral

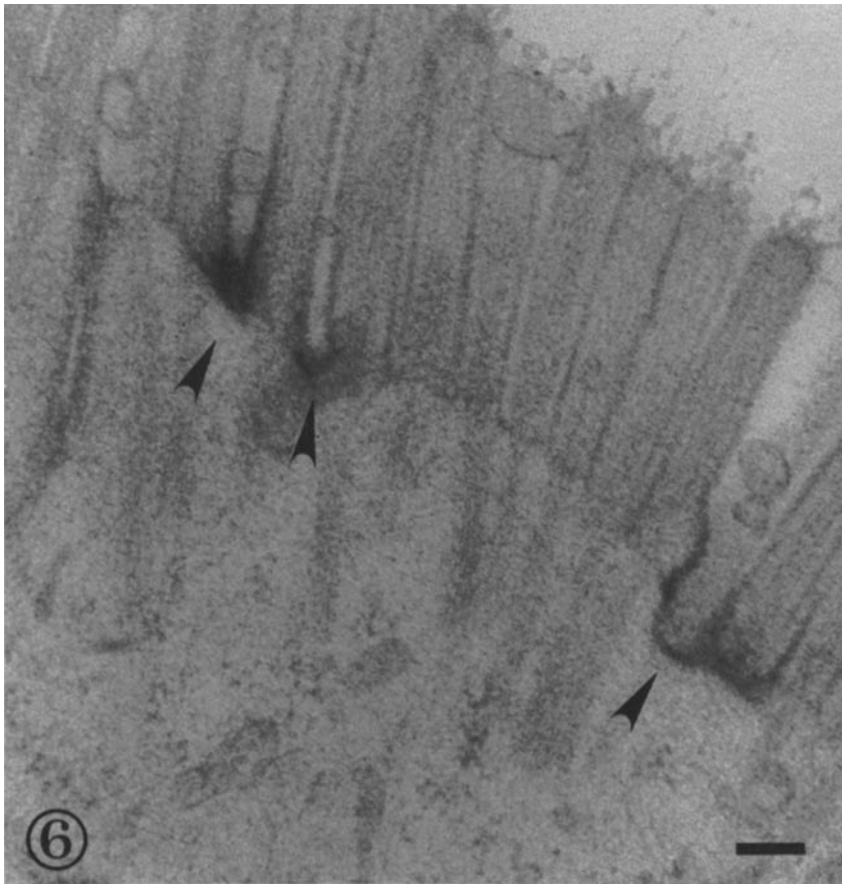


FIGURE 6 Electron micrograph from the villus tip region of dog ileum that has been reacted with anti-IF-cbl receptor. This section has been counterstained with uranyl acetate and lead citrate in an attempt to demonstrate any "coating" of the microvillus pits that contain immunoreactive receptor (black arrowheads). A coat of electron dense particles was not demonstrated. Bar, 0.2 μ m. \times 45,000.

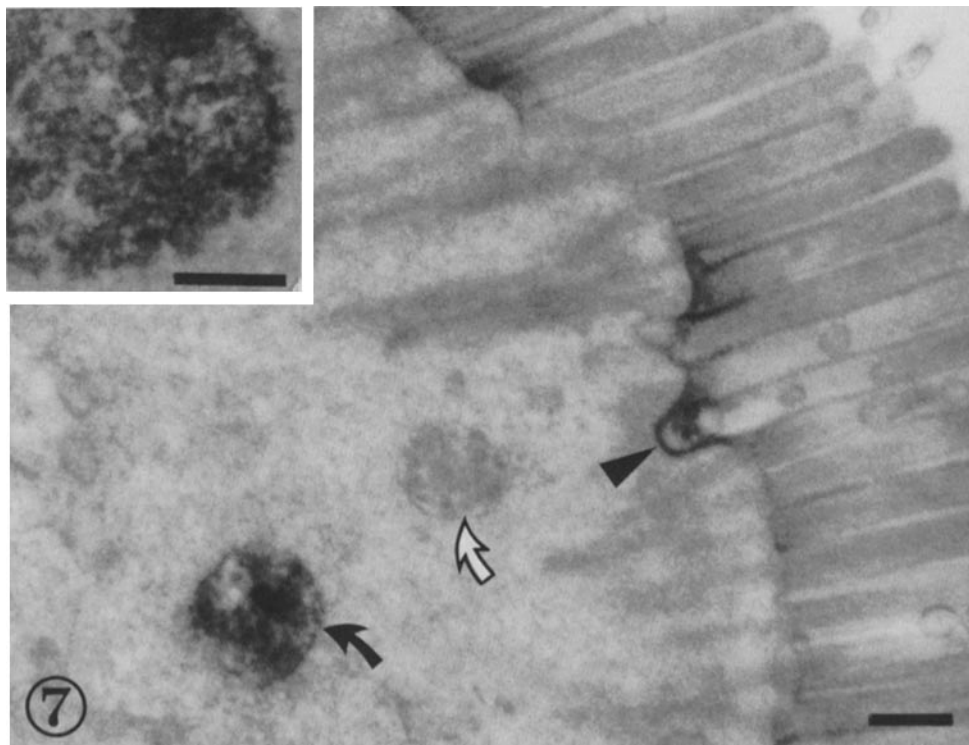


FIGURE 7 Electron micrograph from the villus tip region of dog ileum that has been reacted with anti-IF-cbl receptor. Immunoreactive receptor is associated with microvillus pits (black arrowhead), and a multivesicular body (black arrow). In the inset, at higher power, it is apparent that the receptor is present on both the surface membrane and the small intraorganelle vesicles. The majority of multivesicular bodies seen in these cells did not contain receptor (white arrow). Bars, 0.2 μ m. \times 54,000; \times 76,700 (inset).

TABLE II
Intra- and Intercellular Distribution of IF-cbl Receptor

	RER	Golgi	Smooth vesicles	Microvillus pits (%)	Perinuclear membrane	Multivesicular bodies
Crypt	Rare	—	—	—	—	—
Mid-villus	40	Rare	40	10-20	—	—
Villus tip	50	Rare	90-100	90-100	70	10

Rare, 0-2%; None, —. Percent refers to the percent of cells visualized that contained a specifically stained organelle. Values are rounded off to the nearest 10%.

surface membrane. The unique capability of immunocytochemical studies to define this intra-villus variability will be important as an adjunct to biochemical studies attempting to quantitate mucosal receptor in normal and pathological states.

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