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RECEPTOR-MEDIATED ENDOCYTOSIS OF COBALAMIN (VITAMIN B_{12})

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ABSTRACT

Dietary cobalamin (Cbl) (vitamin B_{12}) is utilized as methyl-Cbl and the coenzyme 5′-deoxyadenosyl Cbl by cells of the body that have the enzymes methionine synthase and methyl malonyl CoA mutase, which convert homocysteine to methionine and methyl malonyl CoA to succinyl CoA, respectively. Prior to conversions and utilizations as the active alkyl forms of Cbl, dietary Cbl is absorbed and transported across cellular plasma membranes by two receptor-mediated events. First, dietary and biliary Cbl bound to gastric intrinsic factor (IF) presented apically to the ileal absorptive enterocytes is transported to the circulation by receptor-mediated endocytosis via apically expressed IF–Cbl receptor. Second, Cbl bound to plasma transcobalamin (TC) II is taken up from the circulation by all cells via a TC II receptor expressed in the plasma membrane of these cells, and in polarized cells via a TC II receptor expressed in the basolateral membranes. This review updates recent work and focuses on (*a*) the molecular and cellular aspects of Cbl binding protein ligands, IF and TC II, and their cell-surface receptors, IF–Cbl receptor and TC II receptor; (*b*) the cellular sorting pathways of internalized Cbl bound to IF and TC II in polarized epithelial cells; and (*c*) the absorption and transport disorders that cause Cbl deficiency.

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CONTENTS

INTRODUCTION

Cobalamin (Cbl) (vitamin B_{12}) is an essential micronutrient required for the health and well-being of all higher animals, including man. However, because of its highly polar nature, Cbl cannot traverse cellular plasma membranes when present in physiological amounts. Thus, all higher animals use two hydrophobic protein ligands, intrinsic factor (IF) and transcobalamin (TC) II, present in gastric juice and plasma, respectively, to bind and transport Cbl across the cellular plasma membranes. Initially, dietary Cbl bound to food proteins is extracted by the concerted action of acid and proteases in the stomach and by pancreatic proteases in the intestinal lumen. Cbl liberated is eventually sequestered by IF and transported across the intestine via IF–Cbl receptor (IFCR) and released into the circulation. Cbl bound to plasma TC II is taken up by all other tissues/cells via TC II–Cbl receptor and is utilized as methyl Cbl or Cbl coenzyme.

This review discusses recent studies on the cell and molecular biology of Cbl transport proteins and on the sorting of Cbl following its endocytosis in a polarized epithelial cell. Earlier literature is cited only when pertinent, and a more detailed discussion of these earlier studies can be found elsewhere (23, 75–78). It is hoped that this review provides new insights into the functional aspects of these fascinating proteins and the intricate pathways of Cbl sorting in a polarized cell and that it stimulates further research in this area.

INTRINSIC FACTOR AND TRANSCOBALAMIN II

Synthesis and Regulation of Expression

IF is a secretory glycoprotein secreted in a regulated manner (76, and references therein). In humans, IF is localized mainly to the parietal cells, but it is also detected at the margins of the anatomical regions in clusters of chief cells,

enteroendocrine and endothelial cells (37). In contrast, in rats, IF is mainly localized to the chief cells, but a small percentage of the parietal cells also stain for IF (86). These ultrastructural observations have suggested that IF can be synthesized in more than one gastric cell type in any given species. Multiple gastric cell expression of IF is due to a common cell lineage, and studies using transgenic mice (52) have shown that a common cell lineage exists between the principal cell types in the stomach and that, in mice, IF is maximally expressed in the chief cells. The molecular mechanism of IF gene expression during differentiation of the various gastric cell types is not known. When a fusion gene consisting of a nontranscribed mouse IF gene and human growth hormone gene ($IF^{-1029\ to\ +55}/hGH^{+3}$) was expressed in mice, the transgene was expressed in the parietal but not in the chief cells (55). These results indicate that the *cis-trans* interactions involved in regulating cell-specific expression of IF are different, because the construct used in these studies lacked either the *cis*-elements required for IF gene expression in the chief cell or its suppression in the parietal cell.

In contrast to humans, in rats and mice, where IF expression is limited to the stomach, the IF gene is transcribed by the canine pancreatic duct cells (87), and IF activity has been detected in opossum pancreas (66). These observations suggest that cellular factors responsible for IF gene transcription in tissues vary across species. In addition to tissue- and cell-specific regulation of the IF gene, expression of it is also regulated in rat stomach by cortisone (20) and by growth hormone (54). Taken together, these studies indicate that IF gene expression is regulated by factors that are intrinsic to the cell, and by extrinsic factors as well.

TC II is synthesized by many cell types in culture (76, and references therein) and is secreted in a constitutive manner. Steady state TC II mRNA measurements revealed that TC II gene is expressed in many human and rat tissues, but in a tissue-specific manner (49). In humans, TC II mRNA levels are highest in the kidney and could arise from a mixed population of cells, mainly endothelial and epithelial cells. When transfected in human epithelial and leukemic cells, human TC II gene promoter is regulated positively by a distal GC-box and negatively by a proximal GC/GT box (51). Both these elements were bound by transcription factors, Sp1 and Sp3, and cotransfection studies using Sp1 and Sp3 expression plasmids revealed that although Sp1 activated the weak promoter activity of the TATA-less TC II gene, Sp3 suppressed Sp1-mediated transactivation of TC II transcription. These studies have suggested that TC II gene expression in human tissues/cells may be controlled by the relative ratios of transcription factors Sp1 and Sp3 that bind to the GC/GT box and that the weak promoter activity is due to transcriptional suppression caused by the binding of Sp3 to the proximal GC/GT box.

Structure, Function, and Genomic Organization

Human IF and TC II are single polypeptides of approximately 43 kDa that contain 1 Cbl binding site/mol. Mature IF is larger, 50 kDa, which is due mainly to N-glycosylation. TC II is not glycosylated, as its one site with potential for N-glycosylation (48) is not utilized (84) because of the presence of sequence NPT, a sequence not favorable for glycosylation (42). The carbohydrate on IF appears to have no role in either Cbl or receptor binding, but there is evidence it may have a role in conferring partial or full protection to proteases (28). This is not surprising considering the ability of IF to remain structurally and functionally intact, even after exposure to proteases of the stomach and intestinal lumen.

Monoclonal antibodies to IF (88) and TC II (59) have identified in both IF and TC II two separate binding sites for Cbl and their respective cell-surface receptors. The receptor binding site of rat and human IF has been localized to residues 25–62 at the NH_2 terminus (89). Within this short stretch, the minimum length of the receptor binding is limited to consecutive residues, 25–44. However, when unlabeled IF peptides generated within residues 1–44 were added as competitors, or when mutations were introduced in the IF molecule between residues 25–44, binding to IFCR was not affected (92). These observations suggest that additional receptor binding sites may exist on IF that are exposed after the initial binding of the amino-terminal end to IFCR. It will be interesting to test whether the receptor recognition of TC II is also localized to the amino terminus.

Although both IF and TC II are recognized by two distinct cell-surface receptors, they share a common property of high-affinity Cbl binding. Sequence alignment (Figure 1) has revealed some interesting features of the primary structures of rat IF (21), human TC II (58), and haptocorrins, another type of mammalian Cbl binder (35, 40). Although the overall homology among these Cbl binders is only about 33%, there are six regions of high (60%–80%) homology (Figure 1, *bars I–IV*). These regions are hydrophobic, each containing at least 12 amino acid residues and flanked on both sides by nonconserved hydrophobic regions. Out of 44 residues that are identical in all the Cbl binders, 4 are cysteine and 29 are located in the high-homology regions (Figure 1, *stars*). Many of these regions are rich in residues with small side chains (Ala/Gly) and OH-bearing residues (Ser, Thr, Tyr). When only human IF and TC II sequences are aligned, a similar structural similarity exists, with about 20% of the residues being identical.

The primary structural analyses support the model proposed by Grasbeck (29) for IF binding to Cbl. In this model, CN-Cbl enters into a "pit" of the IF molecule, with its nucleotide portion facing inward and the axial ligand, the CN^- group, facing outward. This model can now be modified based on primary structure analyses to include other Cbl binders. In the modified model (Figure 2),

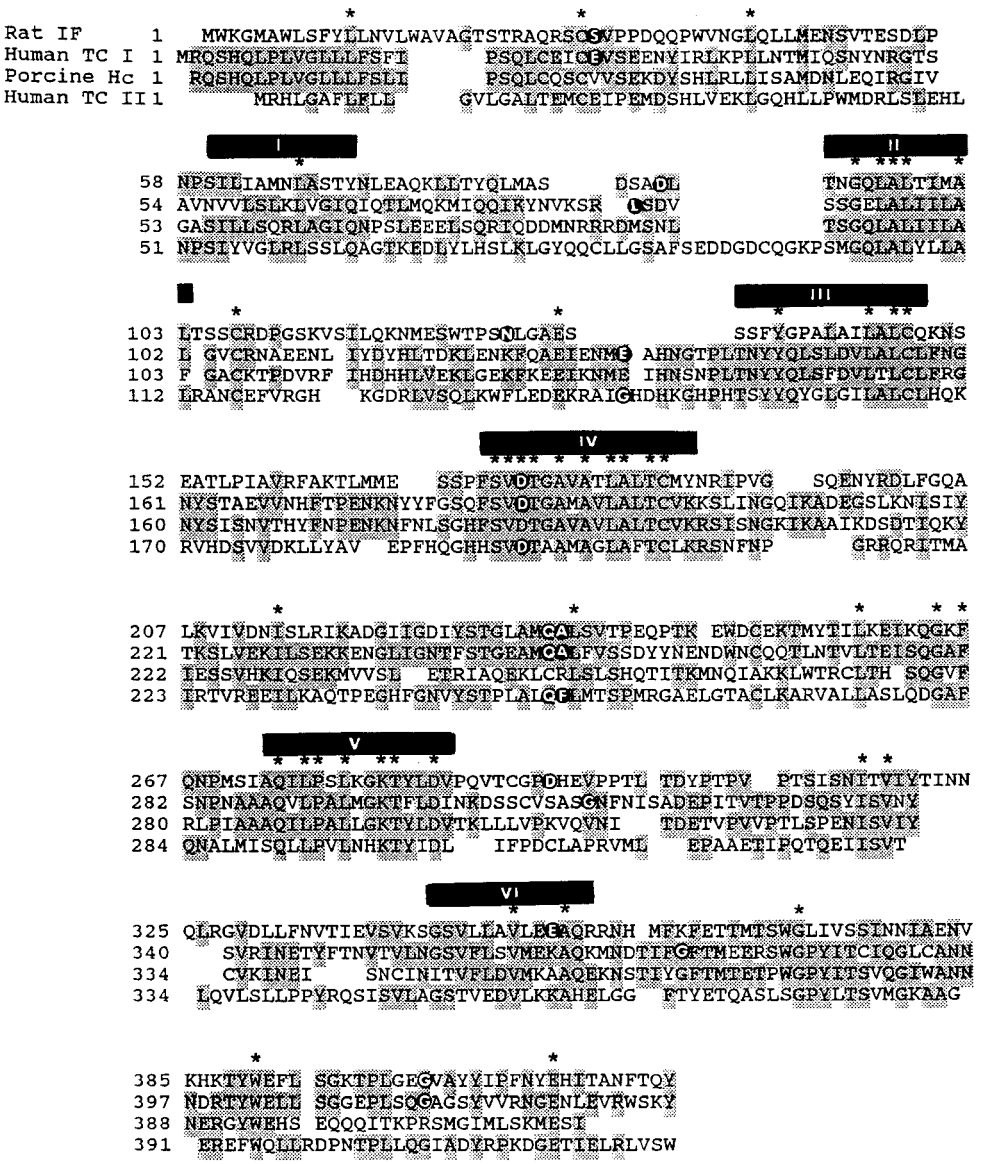

Figure 1 Amino acid alignment of mammalian cobalamin binding proteins. The amino acid sequences of rat intrinsic factor (IF), human transcobalamin (TC) II, and haptocorrins (Hc) (human TC I and porcine Hc) were aligned using the *pileUp program* of the Genetics Computer Group system. (*Bars I–VI*) The higher homology hydrophobic regions; (*stars*) identical residues. (See text for details.)

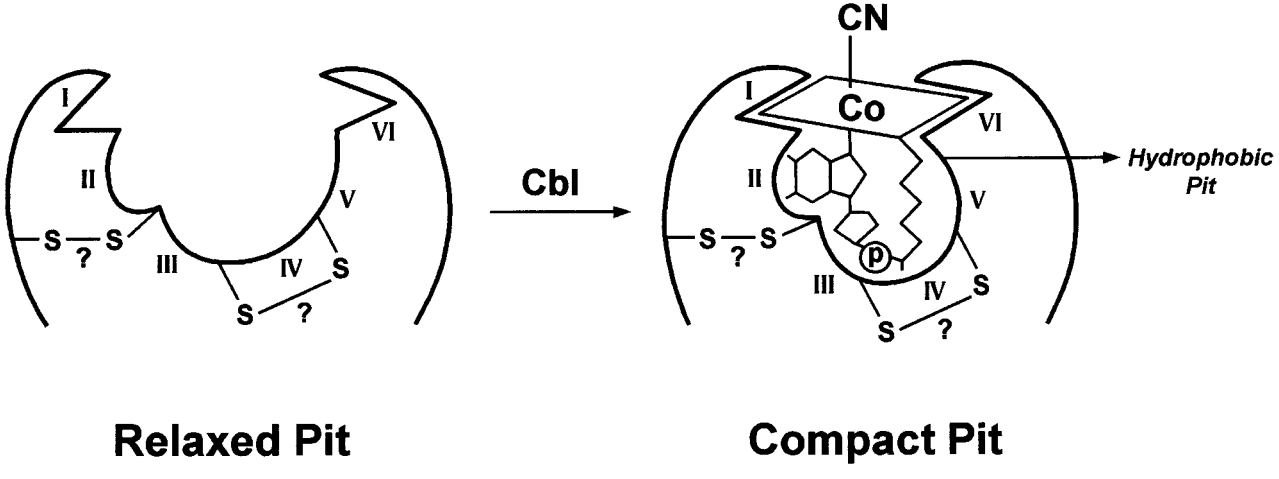

Figure 2 A model for the binding of cobalamin (Cbl) by mammalian Cbl binding proteins. Illustration of the Cbl binding pit before and after Cbl binding. Dimensions of the pit may be defined by one or two disulfide bonds formed by four cysteine residues that are conserved in all Cbl binding proteins.

the interior of the pit will be hydrophobic, involving one or more of the hydrophobic regions. As the nucleotide moiety enters the pit, the nonconserved hydrophilic regions, which may be important in receptor recognition, might be exposed later, after Cbl binding. The hydrophobic pit itself may be conformationally stabilized by one or two S-S bonds, and the presence of four cysteine residues located in an identical position of these proteins supports this suggestion. The occurrence of small (Ala/Gly) and OH-bearing (Ser, Thr, Tyr) residues in this pit may aid in the proper positioning of the nucleotide moiety and in developing hydrogen bonding with the amide groups of the corrin ring. The model proposed for Cbl binding (Figure 2) explains several of the in vitro binding properties of IF and TC II (76), but further in vitro mutagenesis, peptide antibody studies, and X-ray analysis of these proteins are needed to confirm the validity of this model.

IF and TC II also share similarities at the genomic level despite their different locations at chromosomes 11 (36) and 22 (50), respectively. Comparison of the exon size and the boundaries of IF and TC II gene (Figure 3) has revealed similar-sized genes (about 20 kb) that contain in their coding regions an identical number of exons of approximately the same size. Out of the eight exon boundaries, four are conserved. It is interesting that five out of six conserved hydrophobic regions (Figure 1) implicated in Cbl binding are localized to different exons, which suggests that the Cbl binding property of these proteins evolved earlier through gene duplication from a common ancestral gene and that their different receptor binding regions evolved later. The location in chromosome 11 of both IF (36) and haptocorrin (including TC I) (41) genes and in chromosome 22 of TC II (50) suggests that a major event occurred during evolution of Cbl transport proteins that resulted in a possible translocation of TC II gene from chromosome 11 to chromosome 22. Some of the molecular properties of IF and TCII are summarized in Table 1.

INTRINSIC FACTOR AND TRANSCOBALAMIN II RECEPTORS

Tissue Expression and Function

Consistent with its physiological role in the absorption of dietary and biliary Cbl transport, IFCR is expressed in the distal intestine of all mammals except nonplacental mammals, such as the American opossum, a marsupial, in which IFCR activity is high in the more proximal regions (66). These observations suggest that IF-mediated Cbl transport existed as early as the Cretaceous period, some 100–135 million years ago, when marsupials diverged from placenta-containing mammals. In addition, these studies also suggest that the distal

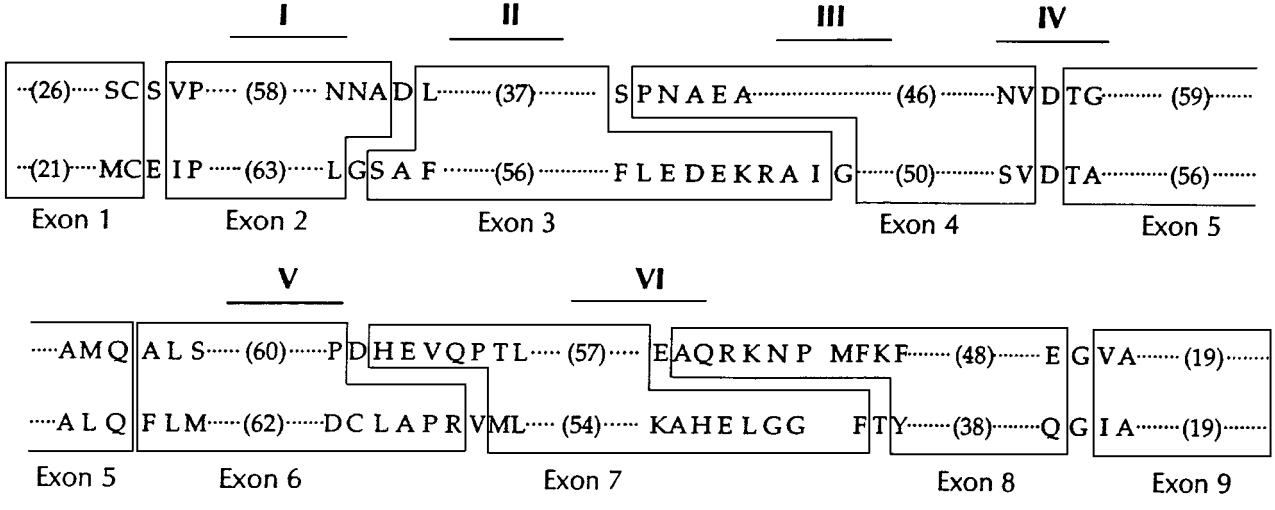

Figure 3 Comparison of the exon size and boundaries of intrinsic factor (IF) and transcobalamin (TC) II. The deduced amino acid sequence of human IF (*top*) and TC II (*bottom*) were aligned by the *pileUP program* of the Genetics Computer Group system. Individual boxes represent the nine exons. The exon number for human IF is started at the coding region. (*Numbers in parentheses*) exon size for the two genes; (*I–VI*) the six conserved hydrophobic regions illustrated in Figure 1.

Table 1 Molecular properties of cobalamin binders[a]

Determinant	Intrinsic factor	Transcobalamin II
Chromosome location	11	22
% Overall homology with each other	33	33
Gene structure	Minimum length 24 kb; 10 exons (60–185 bp) and 9 introns (0.6–4 kb)	20 kb; 9 exons (116–588 bp) and 8 introns (0.35–5.3 kb)
Transcription		
Basal	TATA and CATCCC boxes	TATA and CATCCC-less
Regulatory	ND	GC box(+) and GC/GT box(−)
Receptor recognition site	N-terminal residues 25–44	ND
Inherited disorder	Lack of synthesis or defective synthesis	Lack of synthesis or defective synthesis

[a]ND, Not determined; + and −, positive and negative transcriptional regulation.

(terminal ileum) intestinal expression of IFCR noted in humans may be a later evolutionary event.

In addition to the intestinal mucosa, high levels of IFCR have also been detected in mammalian kidney, including human (82), and its synthesis has been studied in rat kidney (85) and yolk sac (67). The physiological role of renal or yolk sac IFCR in Cbl transport, if any, is not clear, as IF is present only in minuscule amounts in the circulation and is filtered (91). However, because of its apical localization in the kidney (82), it may play a role in the tubular reabsorption of filtered IF, thus preventing urinary loss of Cbl. Supporting this suggestion are studies demonstrating the presence of extremely small amounts of IF bound to the apical brush border membranes of rat kidney (60). Evidence that human renal IFCR may have some additional role has come from studies on patients with selective intestinal malabsorption, generally known as Imerslund-Grasbeck syndrome (30, 39). These patients develop Cbl deficiency and also have proteinuria (31), which is not corrected when parenteral administration of Cbl corrects the intracellular Cbl deficiency and the ensuing megaloblastic anemia (14), or even the usually nonreversible cerebral atrophy associated (74) with this disease.

More direct evidence (81) that renal and yolk sac IFCR may have a role in the general endocytosis of proteins has come from the recent demonstration that IFCR expressed in these tissues is identical to a protein known as gp280, a protein with teratogenic activity localized mainly in the clathrin-coated areas of proximal tubule and yolk sac epithelia (72, 73). Currently, there is no evidence that the teratogenic activity of the IFCR/gp280 in pregnant rats is due to an antibody-mediated inhibition of Cbl delivery to the fetus, as Cbl delivery

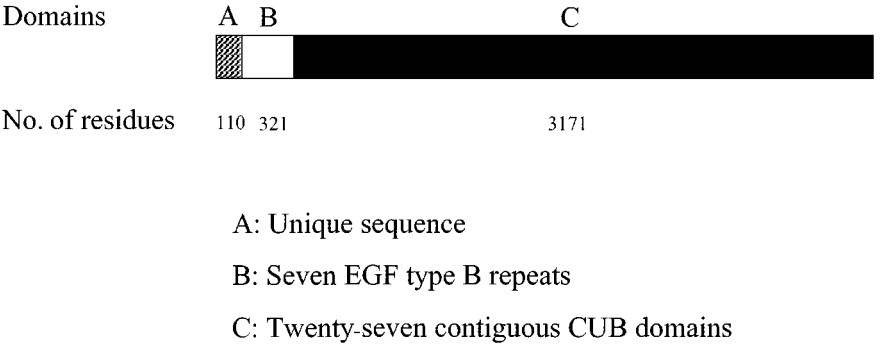

Figure 4 Structural domains of intrinsic factor–cobalamin receptor (IFCR)/gp280. The three domains (*A–C*) of IFCR/gp280 based on the cDNA predicted sequence is illustrated. EGF, epidermal growth factor–like domanins; CUB, cubulin.

to the fetus from the maternal circulation is thought to occur bound to TC II. However, studies using cultured yolk sac BN/MSV cells have shown that the IFCR/gp280 antibody mediates disruption of general endocytic trafficking events (44). Thus, it is likely that both renal and yolk sac IFCR/gp280 may act as scavenger receptors in the general apical endocytic process, and a similar role for intestinal IFCR may also exist, as the ileum is a site of high endocytic activity.

Recently, a cDNA clone has been isolated from rat yolk sac carcinoma BN/MSV (57) cDNA libraries. The cDNA-predicted amino acid sequence revealed a polypeptide of 396,953 Da with three distinct domains (Figure 4), called cubulin. The predicted sequence of cubulin lacks a transmembrane domain, which is consistent with encoding proteins that would have a plasma membrane topology of a peripheral protein (57) and which explains some aspects of IFCR/gp280 expression and its molecular properties. These include the following: (*a*) Cubulin cDNA recognizes a single transcript of 11.6 kb in the intestine, kidney, and yolk sac tissues, where IFCR/gp280 is expressed; (*b*) antiserum to cubulin recognizes a single protein of 460 kDa in the intestinal, renal, and yolk sac membranes, the same size as rat renal IFCR (457 kDa), determined using quantitative amino acid analysis (82); (*c*) what the peripheral topology is of intestinal IFCR, as determined by the ability of various reagents such as chaotropic salts, detergents, and phospholipases to release IFCR following its reconstitution in artificial lipid bilayers (79).

Taken together, these studies show that IFCR/gp280 and cubulin are identical. Earlier estimates, based on mobilities on sodium dodecyl sulfate–

polyacrylamide gel electrophoresis, of the sizes of 230 kDa and 280 kDa for IFCR and gp280, respectively, were only approximations. Because IF is the only physiological extracellular ligand known to bind to IFCR with high affinity, the change in the nomenclature of IFCR to cubulin (57), a change based on its structure and not its function, is probably not needed at this time, and thus, at least for the purposes of this review, the term IFCR is maintained. Earlier limited proteolysis studies of canine IFCR (80) and hog IFCR (93) identified IF-Cbl binding to a receptor fragment of approximately 70–80 kDa, almost the same size as the polypeptide found in human urine that bound IF-Cbl (71). However, the location of the functional 70- to 80-kDa region of IFCR has not been determined. Additional studies are required to confirm that cubulin actually encodes functional IFCR and to identify which of the three regions of IFCR bind to IF-Cbl.

In contrast to IFCR, TC II–Cbl receptor (TC II–R), because of its role in promoting plasma transport of Cbl to all tissues/cells, is expressed in many tissues. Immunoblot studies have shown that TC II–R protein is expressed in many human (12) and rat tissues (11) in a tissue-specific manner and that it is highest in human kidney and in the intestine/kidney of rats. In rats, TC II–R activity and protein levels, and tissue transport of plasma Cbl, are regulated by cortisone (11). In addition, unlike IFCR, TC II–R is not regulated during postnatal development of the intestine and kidney, and in the intestine, its expression is uniform throughout the entire length of the gut (11). These studies have underscored the importance of TC II–R in mediating plasma transport of Cbl to all tissues/cells throughout postnatal development, and one study (13) has shown the binding of TC II–Cbl to germ cells, indicating that TC II–R is a germ cell product.

In addition to TC II–R, whose role in plasma transport of Cbl is well established, another protein known as megalin has been shown to bind to TC II–Cbl in vitro and mediate its endocytosis in BN/MSV cells and perfused renal proximal tubules (56). Megalin, a 600-kDa endocytic receptor, binds to a large number of unrelated ligands (16). The physiological significance, if any, of a megalin-mediated TC II–Cbl uptake system in the plasma transport of Cbl is not evident, for the following reasons. First, plasma TC II–Cbl uptake occurs in all cells, not just in epithelial cells, where megalin expression has been detected (16). Second, adrenalectomy in rats inhibited the plasma transport of absorbed Cbl to the kidney, and this inhibition is due to loss of a single 124-kDa TC II–R activity and a single 124-kDa protein in the plasma membranes of kidney (11). It is not known whether cortisone deficiency affects megalin expression. Third, megalin, an intracellular endocytic receptor, is confined to the apical (16), but not the basolateral, membranes. Fourth, circulating antibodies

to pure human placental TC II–R cause Cbl deficiency in rabbits (9). Fifth, the harvested antiserum raised to TC II–R inhibited TC II–Cbl uptake as well as the incorporation of [^{57}Co]Cbl into Cbl-dependent enzymes when added to the basolateral side of polarized intestinal epithelial Caco-2 cells (9). Finally, TC II–R antiserum recognizes a single band of 124 kDa, but not 600 kDa, in multiple human (12), rat (11), and rabbit (9) tissue membranes, including kidney. Thus, it is highly unlikely that megalin plays any significant role in plasma transport of Cbl. However, because of its apical expression in the kidney proximal tubules, renal megalin may function in the tubular reabsorption of TC II–Cbl, thus preventing Cbl loss in the urine.

Synthesis, Processing, and Trafficking

IFCR/gp280 is synthesized in a number of epithelial cells (3, 32, 61, 63). Epithelial Caco-2 cells, although derived from human colon adenocarcinoma, differentiate as enterocytes and synthesize IFCR, with peak levels of expression occurring after reaching confluence (63). Because there are higher levels of expression of IFCR in opossum kidney (OK) cells (83), some aspects of its synthesis and processing have been studied using these cells. Domain-specific biotinylation and metabolic studies using polarized OK cells have shown that IFCR expression occurs only in the apical brush borders. Expression of IFCR at this site is disrupted by the microtubule-active drugs colchicine and nocodazole, indicating the requirement of an intact microtubular network (61) for its apical expression. Posttranslational processing of IFCR in these cells involves maturation of its N-linked oligosaccharides to the complex type and palmitoylation (62). Its turnover (T) in OK cells is slow, with a $T_{1/2}$ of 48 h (62), which is reduced to 24 h when cells are treated during pulse-chase labeling experiments with tunicamycin, an inhibitor of N-glycosylation. These results have indicated that core N-glycosylation of IFCR is important for its intracellular stability. Processing of N-linked sugars of IFCR/gp280 appears to be dependent on the cell line, as maturation of N-linked sugars of gp280 expressed in BN/MSV cells appears to occur in the Golgi after endocytosis from the plasma membrane during its recycling (3).

Studies using polarized intestinal epithelial Caco-2 cells have provided important information, particularly on the synthesis, turnover, and polarized distribution of TC II–R (6, 7, 10). At steady state, TC II–R is distributed between the basolateral and apical membranes of Caco-2 cells in the ratio of 6:1, similar to that observed using basolateral and apical membranes isolated from human intestinal mucosa (7). Metabolic labeling and domain-specific biotinylation studies have shown that the targeting of TC II–R to the basolateral membranes is a rapid process (10) and is regulated by post-*trans* Golgi (TGN) trafficking.

Disruption either of the Golgi by the macrocyclic antibiotic brefeldin A (6) or of disulfide bond formation of TC II–R by treating Caco-2 cells with low concentrations of N-ethylmaleimide (10) inhibited its post-TGN trafficking but not its transport from the endoplasmic reticulum to the TGN.

One interesting aspect of TC II–R trafficking to the basolateral surface of Caco-2 cells is its ability to change its physical state from that of a monomer (62 kDa) to that of a dimer (124 kDa) (6). TC II dimerization occurs only in the basolateral membranes, and not in the Golgi or the TGN, and is due to its selective interaction with cholesterol-rich (>10 mol%), highly ordered membrane (6) domains of the basolateral membranes. Lack of TC II–R dimerization in the endoplasmic reticulum and the TGN is due to low (<10 mol%) levels of cholesterol in these intracellular organelle membranes. In vitro, the physical state of TC II–R can be converted in situ in isolated tissue membranes, from one form to the other, by manipulating their cholesterol content (8). The functional significance of dimerization of TC II–R is not known, but the dimers, like the monomers, are functional in ligand binding and internalization.

TC II–R levels are up-regulated in rapidly proliferating leukemia cells (1, 53), transplanted sarcomas (17), and methionine-dependent P 60 glioma cells (24). Taken together, these studies suggest that TC II–R levels are regulated by a network of intracellular events, which could include intracellular levels of Cbl, more particularly Me-Cbl. When the demand for Cbl increases during rapid proliferation, these cells up-regulate TC II–R to import more Cbl. Further studies are needed to understand the cellular and molecular mechanism by which cells regulate Cbl entry by regulating their cell-surface TC II–R expression, and how this process is turned on or off depending on cellular proliferation and differentiation status. Table 2 summarizes what is currently known about the various aspects of IFCR and TC II–R.

RECEPTOR-MEDIATED ENDOCYTOSIS OF CBL

General Considerations

Transport of Cbl bound to IF and TC II has been studied using animal models, but it is studies using polarized epithelial cells that have provided important clues as to how this micronutrient is processed in the cells. Because of their remarkable ability to mediate endocytosis from both plasma membrane domains, polarized epithelial cells have been used to study endocytosis of Cbl bound to IF and TC II. These cells use the process of bidirectional endocytosis either to transport Cbl out of the cell when internalized bound to IF from the apical side or to retain it and utilize it as Cbl coenzymes when internalized bound to TC II from the basolateral side. This unique property is reminiscent of the in vivo situation

Table 2 Expression and properties of cobalamin receptors[a]

Determinant	IFCR	TC II–R
Tissue expression	Intestine, kidney, yolk sac	All tissues
Developmental regulation	Yes	No
Regional expression in the intestine	Yes (ileum)	No (uniform)
Hormonal regulation	ND	Cortisone
Size	460,000 Da	62,000 Da (monomer)
Glycosylation	Heavily N-linked	Heavily O-linked
Subunits	None	None
Dimerization	ND	Functional Dimer
Minimum size required for ligand binding	70,000 Da	ND
Other ligands	RAP, megalin	None
Other names	gp280	None
Acylation	Palmitoylated	None
Expression in polarized cells	Apical	Basolateral
Turnover ($T_{1/2}$)	48 h	8 h
Recycling	Yes	Yes

[a]IFCR, Intrinsic factor–cobalamin receptor; TC II–R, transcobalamin II receptor; ND, not determined.

where the absorptive enterocytes transport exogenous Cbl (diet) presented to the intestinal lumen bound to IF, to provide Cbl to other cells of the body, and derive Cbl for their own use from the endogenous sources (circulation) bound to TC II.

IF-Mediated Cbl Endocytosis

IF-mediated Cbl endocytosis has been studied using both in vivo animal models and a number of different polarized epithelial cells (76, and references therein). Common findings in these studies include (*a*) apical endocytosis of IF-Cbl, (*b*) degradation of IF, (*c*) intracellular formation of TC II-Cbl, and (*d*) considerable (6–8 h) delay in Cbl transcytosis. Studies using Caco-2 cells (19, 63) have shown that the $T_{1/2}$ of IF degradation (19) and TC II-Cbl formation (63) is about 4 h, indicating that formation of TC II may be a rate-limiting step in Cbl transcytosis. Despite these similarities between the in vivo animal and cell culture studies, one report (19) has shown that in Caco-2 cells, IF-Cbl is also taken up from the basolateral side. This result is surprising, as IFCR expression has not been demonstrated in the basolateral side of polarized cells (61) or in intact kidney (82) or gut (45). It is possible that the basolateral endocytosis of IF-Cbl noted in this study is due not to IFCR but to the use of a different batch

of Caco-2 cells, to incomplete achievement of polarity of filter-grown cells, or even to the distinct possibility that the uptake is nonspecific.

In vivo (68, 69), chloroquine is a lysosomotropic agent that decreases the amount of Cbl transferred to TC II, thus increasing the amount of Cbl retained on IF. In vitro, both leupeptin and ammonium chloride (65) inhibit the degradation of IF, which is thought to be mediated by cathepsin L (27). Taken together, these studies suggest that both the acid pH and the proteolysis of IF are essential for Cbl release and that IF-Cbl is processed by the classical endosomal-lysosomal pathway. Direct evidence for this suggestion has been provided by light and electron microscopic autoradiography studies, which revealed the movement of Cbl from the endosomal invagination to the lysosomes following microinjection of IF-[^{57}Co]Cbl into single proximal tubule (5) in approximately 20 min.

One unresolved issue during IF-mediated Cbl transcytosis is the site of the formation of TC II–Cbl complex. Intracellular localization of TC II in polarized epithelial cells is difficult because of the low levels of its synthesis and the rapid constitutive secretion. Its location in the lysosomes is unlikely, as apo- or holo–TC II would be degraded. Thus, the most likely cellular location of TC II is a vesicle that is distinct from the lysosomes. It is known that following synthesis, TC II is targeted for rapid secretion via the basolateral membranes in polarized epithelial Caco-2 (63) and OK (64) cells, and it is likely that the site of TC II–Cbl formation during IF-mediated Cbl transcytosis occurs in these vesicles. Cbl that is transported out from either the pre-lysosomes (late endosomes) or the lysosomes may enter such a vesicle. The formation of TC II–Cbl complex in a distinct compartment explains the effects of alkalization or inhibition of IF degradation on the decreased Cbl binding by TC II, as the amount of free Cbl transported out of the lysosomes would decrease. This also explains the increased amount of Cbl bound to IF. However, what is not clear in this scenario is how Cbl gains access to TC II. It could occur by simple diffusion of free Cbl, or by fluid-phase transfer during a potential fusion event involving late endosomes or lysosomes with the TC II–containing secretory vesicle.

TC II–Mediated Endocytosis of Cbl

TC II–mediated uptake of plasma Cbl has been shown in a number of cells (34), and the Cbl transported into cells via plasma membrane TC II–R is utilized as Cbl coenzymes. However, in polarized epithelial Caco-2 cells, TC II–R is expressed in both the basolateral and the apical membranes (ratio 6:1), and TC II is able to mediate Cbl endocytosis in proportionate amounts from both directions, but with one difference. Following basolateral endocytosis of TC II–Cbl, Cbl is retained and utilized as Cbl enzymes, whereas apically internalized TC II-Cbl is transcytosed intact bypassing the lysosomes (7).

The apical pathway for TC II-Cbl endocytosis noted in Caco-2 cells also appears to be functional in intact rat intestine, as orally administered labeled TC II appeared intact in the circulation (7). However, the relevance of this pathway in vivo is not evident, as the presence of TC II in the intestinal lumen has not been demonstrated, and patients with either IF or IFCR defects do develop Cbl deficiency (18, 23). On the other hand, there is enough TC II–R activity in the apical brush border membranes (11, 12) that it can be exploited to transport Cbl in patients who, from a variety of inherited disorders (23) or secondary causes (76), malabsorb Cbl. A TC II–deficient child who malabsorbed Cbl had improved absorption of Cbl when TC II was administered orally along with IF-Cbl (4). Further studies are required to assess the potential use of apical TC II–Cbl as a possible Cbl delivery system. The proposed cellular sorting pathways of Cbl bound to IF and TC II in polarized epithelial cells are illustrated in Figure 5.

INHERITED AND ACQUIRED DISORDERS OF CBL TRANSPORT

Human Cbl deficiency occurs as a result of (*a*) defects in the transport proteins (IF, TC II, IFCR), (*b*) defective passage of Cbl through the ileal cell (Cbl F), or (*c*) a number of acquired disorders due to aging, surgery, and other diseases (76). Transport disorders of Cbl are inherited as autosomal recessive traits based on genetic criteria and occur in different forms (18, 23).

The molecular basis for defective IF expression is not known because of the rarity of these disorders, and because of the difficulty in obtaining gastric biopsies from otherwise normal patients. Southern blotting of genomic DNA obtained from a patient with pernicious anemia revealed normal restriction fragments, which suggests that in congenital pernicious anemia, major deletions of IF gene had not occurred (36). As the presence of IF mRNA was not tested in these studies, it is not possible to ascertain whether the lack of IF in congenital pernicious anemia patients is due to a transcriptional defect.

The most common form of human TC II deficiency, lack of immunoprecipitable TC II in plasma, has been delineated by the reverse transcriptase–polymerase chain reaction technique using RNA isolated from cultured fibroblasts of TC II–deficient patients. In these patients, TC II is not synthesized because of a great reduction in its TC II mRNA levels that cannot be detected even by the sensitive ribonuclease protection assays (49). Sequencing of eight mutant alleles, from four patients, amplified by reverse transcriptase–polymerase chain reaction revealed that the mRNA deficiency is due to heterogeneous forms of mutations and that these mutations are private and occurred in individual families. The mutations of TC II in these patients include small (four nucleotide) and

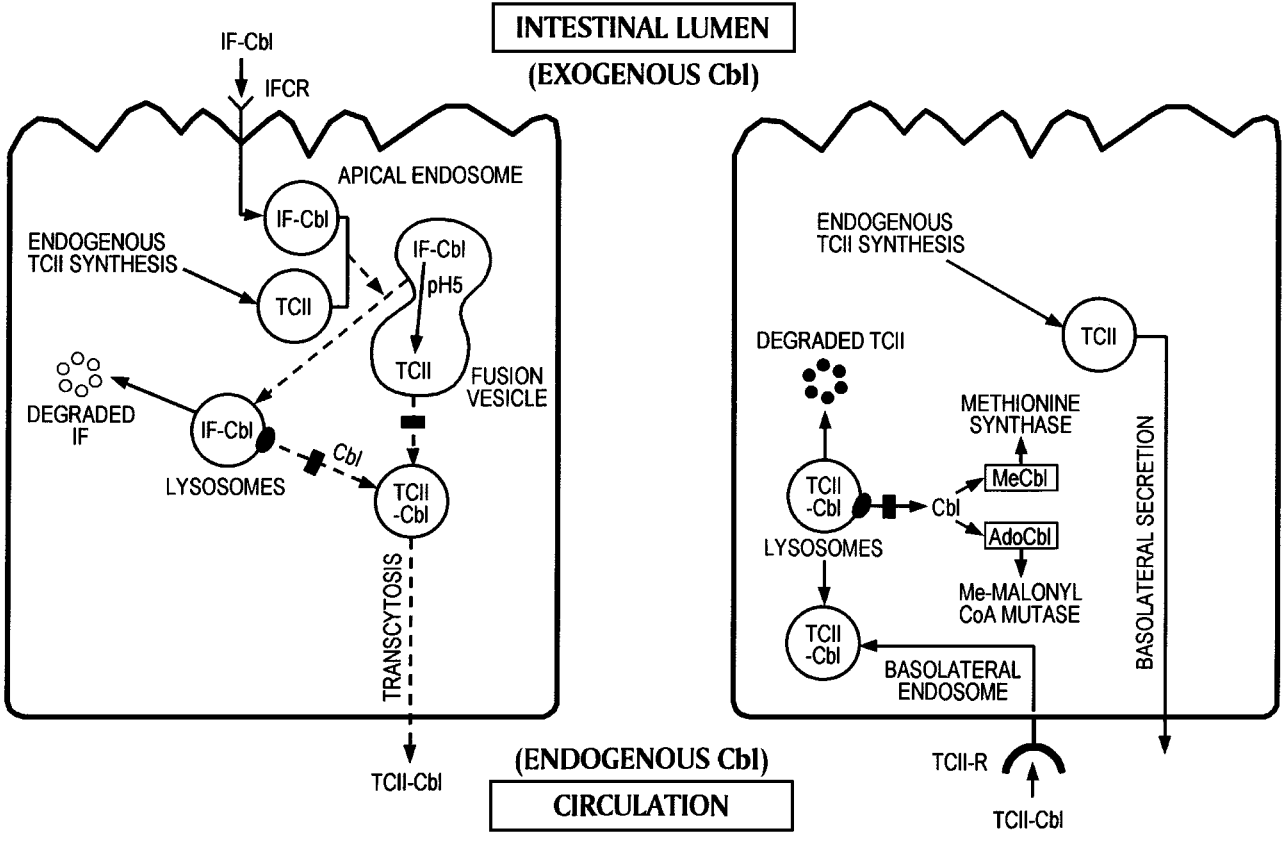

Figure 5 Proposed cellular sorting pathways of cobalamin (Cbl) in a polarized epithelial cell. (*Left*) The transcytosis of Cbl following the apical internalization of Cbl bound to intrinsic factor (IF). (*Broken lines*) Incompletely defined pathways. (*Right*) Retention and utilization of Cbl internalized from the basolateral side bound to plasma transcobalamin (TC) II. (*Dark ovals*) The lysosomal transporter of Cbl; (*dark rectangles*) the block in Cbl sorting in Cbl F patients. IFCR, Intrinsic factor–cobalamin receptor. (See text for details.)

Table 3 Inherited and acquired causes of cobalamin (Cbl) malabsorption[a]

Disorders	Pathophysiology
Inherited	
Lack of luminal IF	Cbl remains bound to Hc and not recognized by IFCR
Lack of IFCR at brush border	Lack of endocytosis of Cbl
Lack of intracellular TC II	Cbl cannot exit the enterocytes
Cbl F (defective lysosomal transporter?)	Cbl retained in the lysosomes
Acquired	
Gastric surgery or gastritis	Cbl release from food proteins impaired due to lack of acid/pepsin
Zollinger-Ellison syndrome	Impaired transfer of Cbl from Hc to IF due to low lumenal pH competition for Cbl
Bacterial overgrowth	Competition for Cbl uptake
Pancreatic insufficiency	Impaired transfer Cbl from Hc to IF due to lack of pancreatic proteases
Surgical resection (Crohn's disease)	Loss of IFCR

[a]IF, Intrinsic factor; Hc, haptocorrins; IFCR, IF–cobalamin receptor; TC II, transcobalamin II.

big deletions (most of the defective allele) of the gene (46), nonsense mutations, and possible transcription defect (47).

In patients with Cbl F, Cbl deficiency occurs as a result of retention of Cbl in the acidic vesicles, endosomes, or lysosomes (70, 90). Transport of Cbl out of these acidic vesicles is important for either its transcytosis bound to TC II (intestinal cells) or its utilization as Cbl coenzymes and is mediated by a Cbl-specific membrane transporter (38). It is not known whether the Cbl transporter is defective in patients with Cbl F.

Patients with selective intestinal malabsorption of Cbl develop Cbl deficiency because of decreased IFCR activity at the cell surface, perhaps caused by the synthesis of an unstable receptor (15, 22, 33). The cellular basis of IFCR deficiency in the human disease appears to be similar to the canine model of the disease (26), where because of its retention (25) inside the cell, IFCR expression in the apical brush border is poor (26). The molecular basis of IFCR deficiency in the human selective Cbl malabsorption syndrome is not known. With the availability of the human IFCR gene (43), which is localized to the same gene locus as the autosomal recessive megaloblastic anemia gene (2), further studies are needed not only to delineate the molecular basis for the development of Cbl deficiency resulting from its malabsorption, but also to further understand its role in causing proteinuria in these patients.

It is important to recognize Cbl deficiency in children early, so that they can be treated to prevent complications leading to the development of megaloblastic

anemia or neuronal damage. In adults, development of Cbl deficiency due to a cause other than strict vegetarianism or intestinal infestations is largely nonreversible. Frequent treatment with large doses (milligrams) causes a small percentage of Cbl to diffuse across the cell membranes to maintain normal cellular function. Table 3 summarizes the pathophysiology of Cbl malabsorption due to various inherited and acquired causes.

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