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Cobalamin transport proteins and their cell-surface receptors

Bellur Seetharam and Raghunatha R. Yammani

The primary function of cobalamin (Cbl; vitamin B_{12}) is the formation of red blood cells and the maintenance of a healthy nervous system. Before cells can utilise dietary Cbl, the vitamin must undergo cellular transport using two distinct receptor-mediated events. First, dietary Cbl bound to gastric intrinsic factor (IF) is taken up from the apical pole of ileal epithelial cells via a 460 kDa receptor, cubilin, and is transported across the cell bound to another Cbl-binding protein, transcobalamin II (TC II). Second, plasma TC II–Cbl is taken up by cells that need Cbl via the TC II receptor (TC II-R), a 62 kDa protein that is expressed as a functional dimer in cellular plasma membranes. Human Cbl deficiency can develop as a result of acquired or inherited dysfunction in either of these two transmembrane transport events. This review focuses on the biochemical, cellular and molecular aspects of IF and TC II and their cell-surface receptors.

Cobalamin (Cbl; vitamin B_{12}) is a water-soluble vitamin of molecular weight 1357 that is present in limited amounts in the diet, primarily in meat, eggs and dairy products. Owing to its highly polar nature, the physiological amounts of Cbl that are needed on a daily basis (2–5 μg) cannot be absorbed by diffusion, an inefficient process. Thus, all higher animals including humans have developed a complex yet efficient mechanism to extract very tiny amounts of Cbl present bound to food proteins, then absorb and transport it into cells.

Two distinct receptor-mediated events are required to absorb and utilise Cbl in mammals (Fig. 1). First, intestinal absorption of dietary and biliary Cbl involves gastric intrinsic factor (IF)-mediated transcellular transport of Cbl across the ileal cell by receptor-mediated endocytosis. The 460 kDa receptor for IF–Cbl (Ref. 1) was originally known as IFCR but is now known as 'cubilin' because of the presence of a large number of CUB domains in its structure (see below) (Ref. 2). After entry into the ileal cell, Cbl is transported across it bound to another Cbl-binding protein, transcobalamin II (TC II) (Ref. 3). Absorbed Cbl bound to plasma TC II is then transported to all cells of the body that need Cbl via portal vein blood (Ref. 4). The second receptor-mediated

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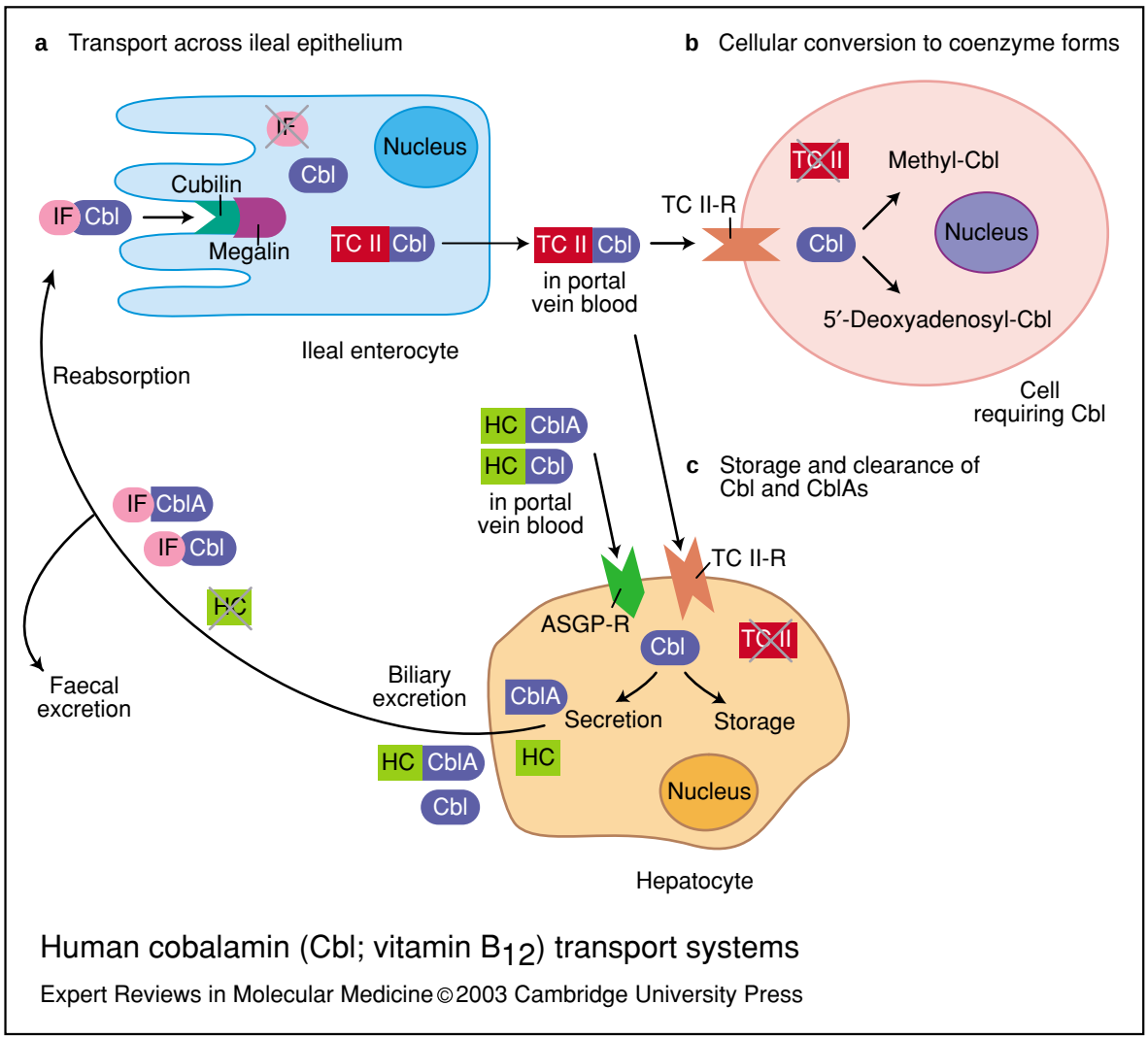

Human cobalamin (Cbl; vitamin B_{12}) transport systems

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Figure 1. Human cobalamin (Cbl; vitamin B_{12}) transport systems. (a) Transport across ileal epithelium. Within the ileum, dietary cobalamin (Cbl) is bound by gastric intrinsic factor (IF), a glycoprotein secreted by the gastric epithelium; the IF–Cbl complex then binds to cubilin, a receptor localised to the apical pole of ileal epithelial cells (enterocytes). Cubilin lacks a membrane-anchoring domain, but is thought to associate with the apical enterocyte membrane through an interaction with megalin. Cbl enters the ileal cell by receptor-mediated endocytosis, and, following lysosomal degradation of IF, is transported across the cell bound to the carrier protein transcobalamin II (TC II), to be secreted into the portal vein. (b) Cellular conversion to coenzyme forms. Plasma TC II–Cbl is taken up by most cells, such as hepatocytes and renal proximal tubular epithelial cells, that express the TC II receptor (TC II-R). Within the cells, Cbl is converted to its coenzyme forms, methyl-Cbl and 5'-deoxyadenosyl-Cbl, which are then used for the enzymatic conversion of homocysteine to methionine and methylmalonyl CoA to succinyl CoA, respectively. (c) Storage and clearance of Cbl and CblAs. Hepatocytes take up Cbl or its analogues (CblAs) bound to HC via the asialoglycoprotein receptor (ASGP-R) and Cbl bound to TC II via TC II-R. Cbl liberated following degradation of HC–Cbl or TC II–Cbl is either stored in the form of Cbl-dependent enzymes or secreted via bile to be reabsorbed. CblA either released from HC or still bound to it is secreted via bile. HC in the bile might originate from the circulation (via uptake by the hepatocytes) or from de novo synthesis by the liver. In either case it is degraded in the intestinal lumen by pancreatic proteases. Once in the lumen, the secreted Cbl and CblA bind to gastric IF and, depending upon the availability of free IF–Cbl binding sites of cubilin, are either reabsorbed as a part of the enterohepatic circulation or excreted in the faeces. The three receptors are shown in their monomeric forms, although cubilin and TC II-R are known to exist as a trimer and dimer, respectively; only one of the two subunits of ASGP-R is shown **(fig001bsw)**.

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event is cellular entry of TC II–Cbl via the specific TC II receptor (TC II-R), a 62 kDa protein that functions as a dimer in plasma membranes of most tissues/cells (Ref. 5). Intracellularly, Cbl is converted to coenzyme forms – methyl-Cbl and 5'-deoxyadenosyl-Cbl – and is utilised for the enzymatic remethylation of homocysteine to methionine and for the conversion of methylmalonyl CoA to succinyl CoA, respectively, which are used in various metabolic processes (Ref. 6).

Analogues of Cbl produced by bacteria or ingested from the diet are also absorbed (although not utilised) by mammalian cells, and this is accomplished via a third transport system. These analogues bind with high affinity to haptocorrin (HC; also referred to as TC I or the R protein) and are cleared via hepatic asialoglycoprotein receptor into bile (Ref. 7). In addition to Cbl analogues, which are probably secreted bound to HC, Cbl is also secreted into the bile as a part of enterohepatic circulation following its mobilisation from the liver. It is likely that this process is designed mainly to recirculate absorbed Cbl obtained from the diet and to provide Cbl for tissues/cells that need it when the exogenous sources of Cbl are inadequate or nonexistent. Once in the lumen, Cbl and its analogues are mostly subjected to reabsorption as a part of the enterohepatic circulation, and when their concentrations exceed the capacity of ileal cubilin to bind and reabsorb them, they are excreted in the faeces.

This review focuses on the transport proteins that are required for normal absorption and transport of Cbl, and how defects in their expression or function, due to either inherited or acquired causes, results in the development of Cbl deficiency. When relevant, structural aspects of HC are discussed, but the structural aspects of the asialoglycoprotein receptor are outside the scope of this review and the reader is referred to Ref. 8.

Physiology and pathophysiology of intestinal absorption of Cbl

The entire process of Cbl absorption begins in the stomach, continues in the intestinal lumen, and is completed in the terminal ileum. The multiple mechanisms involved include gastric secretions of acid, pepsin and IF, pancreatic secretion of proteolytic enzymes, and a specific ileal transport system. The most common cause for the development of Cbl deficiency, other than lack of dietary intake (as in vegans and strict vegetarians), is a failure of gastrointestinal absorption of Cbl. Many aspects of this complex normal physiological process have been learned from patients who failed to absorb dietary Cbl for various reasons.

Initial phases

Cbl in the diet is mostly bound to food proteins and must be extracted from these proteins before it can bind to IF and ultimately be transported to the circulation. This is accomplished in three steps (reviewed in Refs 9, 10). First, most of the food-protein-bound Cbl is released in the stomach by a combined action of acid and pepsin. Second, owing to its higher affinity for Cbl at gastric pH, HC derived from saliva and gastric secretions sequesters Cbl released from the food proteins, preventing it from binding to IF. Third, pancreatic proteases such as trypsin, chymotrypsin and others, proteolytically digest HC–Cbl to release Cbl into the intestinal lumen. Since degraded HC cannot re-bind Cbl, Cbl that is freed from HC then binds to gastric IF (Ref. 11).

Intestinal uptake of IF–Cbl

Even though a large amount of IF is secreted from the stomach and about 8–10 μg of Cbl, representing both dietary and biliary sources, is presented to the enterocytes on a daily basis, the capacity for Cbl absorption is limited to about 1–1.5 μg/day. This limited capacity of IF-mediated Cbl transport in the intestine is due to the restriction of expression of cubilin to the terminal ileum, the limited number of IF–Cbl-binding sites at this location, and the slow turnover of the receptor (Ref. 12). However, since the daily loss of Cbl from the 5 mg of Cbl stored by the human body is estimated from animal studies to be about 0.02% (i.e. 1 μg/day) (Ref. 13), it is likely that the limited capacity of ileal Cbl transport in humans is adequate to compensate for the amount of Cbl lost on a daily basis.

In the ileal apical brush border, cubilin is associated with megalin, another large endocytic receptor, of molecular mass 600 kDa (Fig. 2). The interaction between these two proteins has been suggested to be important for the endocytosis of IF–Cbl (Ref. 14). Although cubilin and megalin are associated with each other during endocytosis of IF–Cbl, it is not known whether these two membrane proteins recycle back to the apical membranes still bound to each other or whether they recycle separately.

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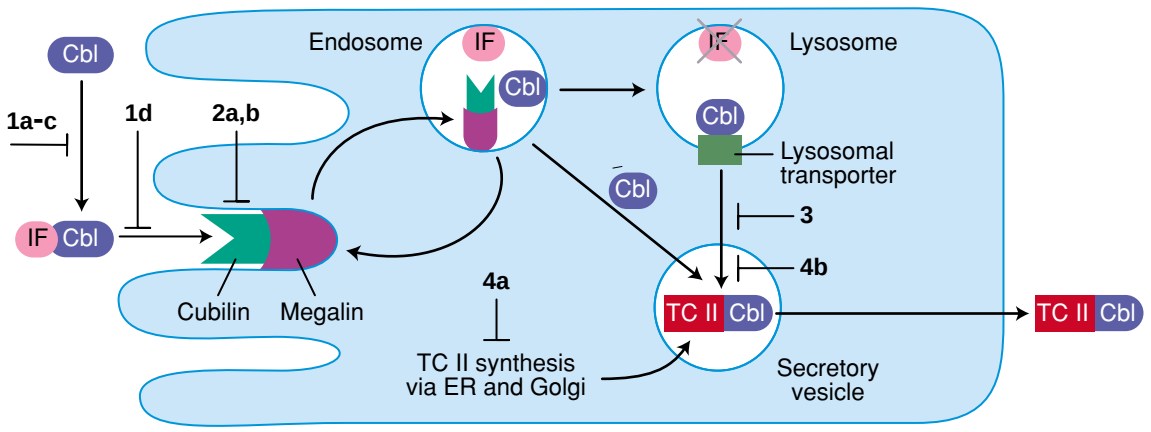

Transcellular transport of cobalamin (Cbl; vitamin B_{12}) in an ileal cell

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Figure 2. Transcellular transport of cobalamin (Cbl; vitamin B_{12}) in an ileal cell. Following binding of Cbl to gastric intrinsic factor (IF) in the gut lumen, the IF–Cbl complex binds to cubilin in the ileal apical brush border. Cubilin is also associated with megalin and the interaction between these two proteins has been suggested to be important for the endocytosis of IF–Cbl. After endocytosis, IF–Cbl–cubilin–megalin is processed via the classical endosomal–lysosomal pathway. The release of Cbl from IF begins in the endosomes as a result of acidic pH alone and is completed as these endosomes mature into lysosomes, and this process requires degradation of IF by lysosomal proteases. Both cubilin and megalin might be recycled, and intralysosomal free Cbl is transported out of the lysosomes. It is speculated that Cbl enters a secretory vesicle that contains transcobalamin II (TC II) as cargo, and TC II–Cbl is then secreted via the basolateral membrane to enter the circulation. There are at least four genetic disorders of Cbl malabsorption known in children, in which blocks occur at specific steps in the transport pathway. The blocks are labelled as types 1–4: type 1 is a lack of formation of the IF–Cbl complex (types 1a–c) or its dysfunction (type 1d); type 2 is a lack of uptake (type 2a) or decreased uptake (type 2b) of IF–Cbl as a result of defects in cubilin; type 3 is retention of Cbl inside the ileal cell as a result of a failure in Cbl transport out of the acidic vesicles; and type 4 is a failure of Cbl to exit the ileal cell and enter the circulation as a result of lack of TC II synthesis (type 4a) or, rarely, dysfunctional TC II that is unable to bind Cbl (type 4b). Abbreviation: ER, endoplasmic reticulum **(fig002bsw)**.

Following binding of IF–Cbl to cubilin, IF–Cbl is endocytosed from the apical or luminal side of the ileal cell, and there is then a delay of about 4 h before Cbl enters the circulation bound to TC II (Fig. 2). Available evidence from several earlier studies indicates that IF–Cbl is processed via the classical endosomal–lysosomal pathway (Ref. 10 and citations therein). At some stage during the endosomal–lysosomal processing of the IF–Cbl complex, IF is degraded and liberated Cbl is transported out of the lysosomes. Although not directly demonstrated, it is speculated that Cbl enters a secretory vesicle that contains TC II as a cargo. TC II–Cbl is then secreted via the basolateral membrane to enter the circulation. The importance of lysosomes during the transcellular transport of Cbl is supported by the evidence that lysosomal inhibitors such as chloroquine or leupeptin prevented IF degradation, resulting in a failure to liberate Cbl bound to it (Refs 15, 16).

Inherited and acquired causes of Cbl malabsorption

Inherited disorders

Cbl malabsorption is known to occur in children as rare inherited disorders, classified as four types. The blocks in transcellular transport of Cbl caused by these inherited disorders are shown in Figure 2 and their pathophysiology is explained in Table 1.

Type 1 malabsorption is defined as lack of formation or dysfunction of the IF–Cbl complex. This can result from: a lack of IF synthesis (type 1a); or synthesis of dysfunctional IF that is

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Table 1. Inherited disorders of cobalamin (Cbl; vitamin B_{12}) malabsorption (tab001bsw)

Type	Definition	Affected molecule	Pathophysiology	Refs
1	Lack of formation or function of IF–Cbl complex due to lack of IF synthesis (1a) or dysfunctional IF (!b,1c,1d)	IF	Cbl is not bound to IF, and both free Cbl and Cbl bound to HC are not recognised by the ileal receptor	6, 94
2	Lack of uptake (2a) or decreased uptake (2b) of IF–Cbl due to defects in cubilin	Cubilin	Entry of Cbl into absorptive ileal cells is reduced or absent	6, 18, 21
3	Failure of Cbl transport out of the acidic vesicles (endosomes or lysosomes)	Lysosomal transporter	Cbl is retained in the ileal cell and thus not available to bind to TC II	24, 28
4	Failure of Cbl exit into circulation due to lack of TC II synthesis (4a) or synthesis of TC II that fails to bind Cbl (4b)	TC II	Cbl fails to exit the absorptive ileal enteroctyes	6, 94

Abbreviations: HC, haptocorrin; IF, intrinsic factor; TC II, transcobalamin II.

sensitive to acid and proteolytic enzymes (type 1b), does not bind Cbl (type 1c), or can bind Cbl but has low affinity for the ileal receptor (type 1d). The molecular basis for expression of these phenotypes is not known. Although serum Cbl levels are usually low, the gastric function and morphology are normal and serum autoantibodies to IF are absent.

Type 2 malabsorption arises from selective gastrointestinal malabsorption of Cbl, and is also known as Imerslund–Grasbeck syndrome (IG syndrome). By linkage analysis, the disease gene of IG syndrome was mapped to a 6 cM region of chromosome 10p12.1 (Ref. 17), the same region that encoded the IF–Cbl-binding protein cubilin. Cbl malabsorption in these patients is due to total lack of uptake (type 2a) or decreased uptake (type 2b) of IF–Cbl as a result of defects in cubilin. Over 200 patients have been identified worldwide, with clusters of cases reported in Norway, Finland and several Middle Eastern countries, including Israel. In addition to megalobastic anaemia and low serum Cbl levels, these patients also have proteinuria, which is not cured even when Cbl status improves with treatment. Malabsorption of Cbl and megaloblastic anaemia noted in Finnish patients have been attributed to two mutations in the cubilin molecule (Ref. 18): a missense mutation resulting in the substitution of a proline for leucine at residue position 1297 of cubilin (FM1); and mutation of a single nucleotide (FM2) that results in the inframe insertion of multiple stop codons in CUB domain 6 (see below). Whereas cubilin in FM1 patients has a decreased affinity for IF–Cbl (Ref. 19), patients with FM2 might have a truncated receptor that fails to reach the apical brush border of ileal absorptive cells.

There is some debate about whether mutations in the cubilin molecule are solely responsible for the Cbl malabsorption and megaloblastic anaemia, as these mutations were noted only in Finnish patients and not in patients from Norway and Saudi Arabia. Studies supporting the role of a protein other than cubilin, which is essential for its functional expression in the apical brush border membranes, have come from a canine model of selective Cbl malabsorption and megaloblastic anaemia (Ref. 20). In these affected animals cubilin was not expressed in both the ileal and renal apical brush border membrane (Ref. 21) and did not contain the disease mutations noted in Finnish patients (Ref. 22). On the basis of these findings, it was suggested (Ref. 22) that more than one phenotype of IG syndrome could exist and that mutations in an accessory protein could affect intracellular trafficking and targeting of cubilin to the apical brush border. Although no such accessory protein has been identified, the

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candidates include receptor-associated protein (Ref. 14), megalin (Ref. 14), and the product of a newly discovered gene, the Amnionless gene (*Amn*) (Ref. 23), that encodes a transmembrane protein. Amn contains a 5'-extracellular cysteine-rich domain, and homozygous mutations in exons 1–4 also lead to both selective Cbl malabsorption and megaloblastic anaemia. However, it remains to be established whether Amn and cubilin gene products function together or independently in Cbl absorption.

Type 3 malabsorption is the intracellular retention of Cbl inside the ileal cell as a result of a failure in Cbl transport out of the acidic vesicles (endosomes or lysosomes). This disorder, also known as Cbl F (Ref. 24), is very rare and has been described in only a handful of patients. Although not characterised well, a lysosomal transporter of Cbl has been demonstrated in rat liver (Ref. 25). Cbl has been shown to accumulate in the lysosomes of Cbl F patients (Ref. 26), and this is thought to arise from the expression of a defective transporter that fails to transport Cbl out of the lysosomes into the cytoplasm. Thus, Cbl cannot bind to TC II, a necessary step before Cbl enters the circulation, and is not available for the cellular synthesis of its coenzyme forms (Ref. 27). A Schilling test (which measures intestinal absorption capacity for Cbl) performed on a Cbl F child showed decreased urinary excretion of Cbl, indicating malabsorption of orally administered Cbl (Ref. 28), and the importance of lysosomes in Cbl transport across the ileal cell. It should be noted that both type 2 and type 3 malabsorption have been collectively known as IG syndrome. In both these disorders there is a failure of Cbl absorption after the formation of IF–Cbl complex and before Cbl is transferred to TC II.

Type 4 malabsorption describes a failure of Cbl to exit the ileal cell and enter the circulation. It results from lack of TC II synthesis (type 4a) or, rarely, dysfunctional TC II that is unable to bind Cbl (type 4b). Surprisingly, children with type 4 malabsorption have normal or nearly normal serum Cbl levels, indicating that most of the circulatory Cbl is bound to plasma HC. However, these children will develop Cbl deficiency as Cbl bound to HC will not be delivered to cells other than hepatocytes because of the paucity of expression of the asialoglycoprotein receptor in many tissues/cells

These genetic disorders of Cbl absorption appear to be inherited as autosomal traits. Many of the disorders have been detected in children residing in western countries, and their incidence worldwide is not known. It is important to diagnose Cbl deficiency in such children as early as possible and treat them with regular intramuscular injections of Cbl to prevent complications that can cause developmental delay and megaloblastic anaemia. These rare genetic disorders are discussed in more detail in other review articles (e.g. Ref. 29).

The molecular basis for the development of Cbl deficiency in children caused by defects in IF and TC II is not fully understood. In a few cases that have been examined, the most common cause of human TC II deficiency, lack of plasma TC II, has been shown to be due to small and large deletions of the gene (Ref. 30) or to nonsense-mutation-mediated mRNA degradation (Ref. 31). In patients with these mutations, no TC II is synthesised. In a few patients who express nonfunctional TC II, the defect appears to be errors in mRNA editing (Ref. 32). In two cousins with juvenile pernicious anaemia, the gene encoding IF was intact suggesting that this rare disease is not due to IF gene deletion (Ref. 33).

Although children with either dysfunction or lack of IF and TC II do develop Cbl deficiency, it is not known whether HC deficiency or dysfunction directly causes Cbl deficiency. HC deficiency has been noted in only a few children and seems to occur either alone or along with a deficiency of other granular proteins such as lactoferrin (Ref. 34). No DNA abnormalities of the gene encoding HC have been noted. Patients with HC deficiency have low serum Cbl levels, but they show no signs of Cbl deficiency, most probably because their plasma TC II levels are usually normal. However, some patients develop myelopathy not attributed to other causes. The role of HC in normal Cbl balance is not known and it is speculated, based on its higher affinity for Cbl analogues than Cbl, that HC might function in the hepatic clearance of harmful analogues of Cbl. Thus, lack of HC might only cause Cbl deficiency when the Cbl analogues accumulate and replace Cbl bound to intracellular Cbl-dependent enzymes and inhibit their function. In addition, given the presence of HC in human milk, it is likely that its absence in milk from HC-deficient mothers might create Cbl deficiency in breast-fed infants (Ref. 29). There is some evidence in animal experiments that in breast-fed pups, Cbl bound to milk HC is taken

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Table 2. Acquired causes of cobalamin (Cbl; vitamin B_{12}) malabsorption (tab002bsw)

Cause	Pathophysiology	Refs
Atrophic gastritis[a]	Loss of parietal cells: lack of IF production	95, 96
Partial or total gastrectomy	Lack of acid production and decreased digestion of food proteins	97, 98
Pancreatic insufficiency	Failure to digest HC–Cbl complex due to lack of pancreatic proteases; HC–Cbl not recognised by ileal receptor	36
Zollinger–Ellison syndrome[b]	Impaired transfer of Cbl from HC to IF because of maintenance of low intraluminal pH	99
Intestinal infestation or bacterial overgrowth	Competition for dietary Cbl by the pathogens	100, 101
Tropical sprue[c]	Flattened villi, causing loss of surface area and loss of cubulin?	102, 103
Ileal disease or surgical resection	Loss of cubilin	104, 105
Hypothyroidism	Decreased cubilin levels?	38
AIDS	AIDS-enteropathy?	106

[a] Chronic inflammation of the stomach with atrophy of the mucous membrane and destruction of the peptic glands.
[b] A tumour of the pancreatic islet cells that causes overproduction of gastric acid, resulting in ulceration at multiple sites (oesophagus, stomach, duodenum and jejunum).
[c] Occurs chiefly in Caribbean, south India and Southeast Asia and is characterised by malabsorption, multiple nutritional deficiencies and mucosal abnormalities in the small bowel. The aetiology is unknown but suggested causes are bacterial, viral or parasitic infection, vitamin deficiency or food toxins.
Abbreviations: AIDS, acquired immune deficiency syndrome; HC, haptocorrin; IF, intrinsic factor.

up by the immature gut, as both IF and cubilin expression occur only after weaning.

Acquired disorders

Malabsorption of Cbl by acquired causes can occur as a consequence of diseases of multiple organs, including the stomach, pancreas, terminal ileum and thyroid (Ref. 10; Table 2). Malabsorption of Cbl caused by diseases of the stomach such as atropic gastritis, and partial or total gastrectomy, involves either injury or loss of parietal cells that produce IF. In addition, because of the lack of acid and pepsin, there might be inadequate release of Cbl bound to food proteins. Patients with gastric tumours (Zollinger–Ellison syndrome) might gradually develop Cbl deficiency as a result of a slow transfer of Cbl from HC to IF. However, these patients appear to absorb crystalline Cbl better than food-protein-bound Cbl (Ref. 35). Exocrine pancreatic insufficiency or intestinal diseases such as bacterial overgrowth, infestation, tropical sprue, ileal disease and Crohn's disease are also known to cause Cbl malabsorption.

Cbl malabsorption in patients with pancreatic insufficiency is correctable with pancreatic supplements (Ref. 36), and in patients with intestinal infestation and bacterial overgrowth it can be corrected following elimination of these causes (e.g. by treatment with antibiotics). Patients with permanent loss of IF following total gastric surgery, or loss of cubilin because of terminal ileal resection, will require Cbl supplementation. If there is sufficient stomach remaining to produce IF or gut remaining to absorb IF–Cbl, Cbl deficiency can be treated with a large dose of oral Cbl; the alternative is regular intramuscular injections of Cbl.

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The mechanism of Cbl malabsorption noted occasionally in patients with hypothyroidism (Ref. 37) is not known and it appears to depend on the severity of the disease. On the basis of animal studies, malabsorption of Cbl in this disorder appears to involve a deficit of the IF–Cbl receptor (Ref. 38).

Structure and cellular expression of the Cbl transporters IF and TC II

Molecular cloning and gene structure analysis of the human Cbl transporters IF, TC II and HC have revealed that these three proteins are derived from a common ancestral gene by gene duplication, despite the localisation of the genes encoding IF and HC to chromosome 11 (Refs 33, 39) and that encoding TC II to chromosome 22 (Ref. 40). It is not known whether the TC II gene was also originally located on chromosome 11 or whether chromosome 22 was originally the location of all three genes. The genes encoding these transporters are of approximately 20 kb length, contain an identical number of exons of approximately the same size, but many of the highly conserved hydrophobic regions of these proteins are localised to different exons. In addition, four out of eight intron–exon boundaries are at identical locations, with the other four located in very close proximity.

Sequence alignment analysis at both cDNA (Refs 41, 42) and genomic levels (Ref. 40) has provided some novel insights into the structure of these proteins. Although the overall homology between IF, TC II and HC is only about 30%, there are several regions in their sequence containing 10–15 residues where the homology is much higher, with nearly 60–80% identity. In addition, these proteins contain conserved cysteine residues at six identical positions. In bovine TC II these six conserved cysteine residues have been shown to form three intramolecular disulphide bonds (Ref. 43) and they appear to influence Cbl binding in vitro. However, more-recent studies (Ref. 44) using in vitro mutagenesis of human TC II and transfection in human fibroblasts have shown that whereas two of the three disulphide bonds, C98–C291 and C147–C187, affected both in vitro Cbl binding and in vivo stability of TC II, the third disulphide bond, C3–C249, had no role in Cbl binding, stability or secretion of TC II. It is likely that, similar to TC II, both IF and HC also form three intramolecular disulphide bonds as these might be required to protect the highly homologous Cbl-binding region(s) from proteolytic degradation.

On the basis of these structural comparative studies, it is very likely that the Cbl-binding regions in these proteins evolved before their different receptor-binding regions. Although the entire IF or TC II molecule might be essential for high-affinity binding of Cbl, binding to their respective receptors might utilise shorter regions within their structures. The initial receptor-binding (cubilin-binding) region of IF is localised to residues 25–62 at the N-terminal region (Ref. 45), where there is very little homology of IF with other Cbl-binding proteins. However, other contact points of IF with its receptor might develop after this initial binding (Ref. 46). This is not surprising since cubilin is a polyvalent protein with multiple structural domains. The receptor-recognition region of TC II has not been identified yet, but preliminary studies indicate that the binding site for TC II-R is localised to the interior of the molecule (B. Seetharam et al., unpublished). It is obvious from these studies that much needs to be learned about the structure–function relationship of these Cbl transport proteins.

TC II, which is not a glycoprotein, is secreted in a constitutive manner from many human cells in culture and in vivo, with endothelial cells appearing to be the primary source of plasma TC II (Ref. 47 and citations therein). TC II is a product of a typical housekeeping gene in that its 5'-flanking region lacks a discernable TATA box, contains multiple start sites and is GC-rich. The differential levels of TC II mRNA noted in tissues (Ref. 48) might be related to relative ratios of the transcription factors Sp1 and Sp3, both of which bind to the GC/GT box (Ref. 49). Additional in vitro transfection studies (Ref. 50) have shown that the physical interactions between Sp1 (which binds to the GC box) and USF1 and USF2 [transcriptional factors of the basic helix–loop–helix (bHLH) family that bind to the E-box from –523 to –528] might play a role in high transcriptional activity of the TC II promoter. In addition to binding USF1 and USF2, the E-box also binds to other members of the bHLH family of transcription factors such as Myc/Max, Mad/Max, and Max/Max, which transactivate many oncogenes. This night partly explain the high plasma TC II levels noted in patients with some forms of cancer (Ref. 47).

By contrast to TC II, which is expressed in many cellular sites, cellular expression of the IF

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glycoprotein is limited to cells derived from tissues originating from the foregut anlage, and its secretion is regulated by a variety of factors (Ref. 9 and citations therein). Sites of IF synthesis include gastric parietal (human), chief (rat) and pancreatic duct (dog) cells. Transgenic mice experiments (Ref. 51) using 1.1 kb of the 5'-flanking region of mouse IF gene fused with the human growth hormone showed transgene expression in the parietal cells, but not the chief cells, although it is the latter that normally express IF in the mouse. These studies have underscored the complexity of cell-specific expression of the IF gene, and further studies are required to identify the cis–trans interactions that are important in activation or suppression of the IF gene in any given type of gastric cell. Hormones such as cortisone (Ref. 52) and growth hormone (Ref. 53) are known to modulate IF mRNA levels in rats; however, it is not known whether the hormones directly affect transcription of the IF gene or exert their effects by altering the stability of IF mRNA. Table 3 summarises important properties of these Cbl-binding proteins.

Structure, function and cellular expression of Cbl receptors

The cubilin receptor

Structure and expression

In the 1970s and early 1980s, a cell-surface receptor for IF–Cbl of molecular mass 200–230 kDa was identified and characterised from the terminal ileal brush border membrane of a number of mammalian species (reviewed in Refs 9, 10 and citations therein). In addition to the ileal mucosa, work in the late 1980s and early 1990s identified high-affinity IF–Cbl-binding sites in the mammalian kidney (Ref. 54) and murine yolk sac (Ref. 55). Several later studies using isolated kidney tissue (Refs 21, 54, 56) demonstrated that the IF–Cbl receptor was synthesised in the kidney and was immunologically identical to its counterpart in the intestine. Moreover, as in polarised intestinal epithelial Caco-2 cells (Ref. 3),

Table 3. Cellular and molecular properties of cobalamin (Cbl; vitamin B_{12})-binding proteins (tab003bsw)

Property	IF	TC II	HC
Cellular expression	Gastric parietal cells Pancreatic duct cells	Endothelial cells Epithelial cells	Granulocytes Renal epithelial cells
Mode of secretion	Regulated	Constitutive	Regulated
Immunological specificity	Distinct	Distinct	Distinct
Polypeptide (kDa)	45	43	46
Number of N-linked sugars	4	None	9 or 10
Carbohydrate (%)	10–12	None	33–40
Cbl-binding site	1	1	1
Number of subunits	1	1	1
Chromosome location	11	22	11
Overall homology with each other	33%	33%	33%
Gene structure	24 kb 10 exons, 9 introns	20 kb 9 exons, 8 introns	12 kb 9 exons, 8 introns
Polymorphism	No	Yes	No

Abbreviations: HC, haptocorrin; IF, intrinsic factor; N-linked, asparagine-linked; TC II, transcobalamin II.

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the apically expressed receptor in polarised opossum kidney cells was able to internalise IF–Cbl and mediate transcellular transport of Cbl (Ref. 57). Furthermore, studies using a family of dogs with selective inherited intestinal malabsorption of Cbl (Ref. 20) demonstrated that the receptor expression in the brush border of both intestine and kidney was inhibited in these animals (Ref. 21). These results initially suggested that both intestinal and renal receptors are under similar types of regulation and thus could be the products of the same gene. The earlier estimates of molecular mass for IF–Cbl receptor in the range of 200–230 kDa were based on its mobility on denaturing electrophoresis – a method that is not accurate for determining the molecular mass of heavily glycosylated proteins whose mass is greater than 200 kDa.

In 1998, an 11.2 kb gene encoding a protein of molecular mass of 460 kDa that bound IF–Cbl with high affinity was cloned (Ref. 2). The linear structure of this protein, cubilin, consists of three structural domains: a 113-residue N-terminal region, followed by eight epidermal growth factor (EGF)-like repeats and a tandemly arranged cluster of 27 CUB domains (Fig. 3). CUB is an acronym for 'complement Clr/Cls, Uegf and bone morphogenic protein-1' (Ref. 2). Each CUB domain consists of about 110 amino acids and such domains are found frequently in developmentally regulated proteins such as bone morphogenic protein (Ref. 58), tolloid protein (Ref. 59) and spermadhesin (Ref. 60).

Ligand interactions

Cubilin is polyvalent in nature and has the ability to bind to a structurally diverse set of ligands (Fig. 4) that include receptor-associated protein (Ref. 1), albumin (Ref. 61), apolipoprotein A-I/high-density lipoprotein (Refs 62, 63), transferrin (Ref. 64), immunoglobulin (Ig) light chains (Ref. 65) and vitamin-D-binding protein (Ref. 66). Although these proteins are normally largely undetected in urine, their levels are elevated in the urine of dogs and humans with IG syndrome.

Two regions of cubilin – the N-terminal 113-residue region and CUB domains 6–8 – are implicated in the binding of both IF–Cbl and albumin. However, since these two ligands do not compete for binding at both the regions, it is likely that the two ligands bind to sites that are spatially distinct in each region (Ref. 67). It is possible that similar spatial differences could exist for the binding of other ligands, such as vitamin-D-binding protein. Indeed, patients with IG syndrome containing a missense mutation, P1297L, in CUB domain 8 that decreases the affinity for IF–Cbl (Ref. 19) failed to excrete vitamin-D-binding protein or 25(OH)-D_3 in their urine (Ref. 66); however, there was an increase in the urinary excretion of vitamin-D-binding protein and 25(OH)-D_3 in patients with an intronic mutation that resulted in the introduction of a premature termination codon and truncation of the cubilin molecule (Ref. 18).

In addition to its ability to bind to IF–Cbl and to albumin, the N-terminal 113-residue region

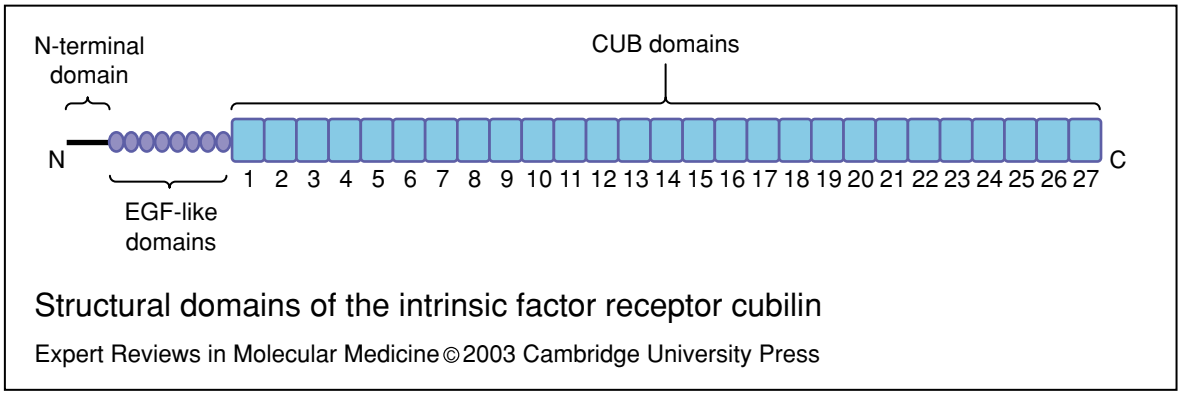

Figure 3. Structural domains of the intrinsic factor receptor cubilin. The three structural domains of cubulin are shown: the 113-residue α-helical N-terminus, implicated in interactions with megalin and possibly the lipid bilayer and in ligand (IF–Cbl and albumin) binding; the eight epidermal growth factor (EGF)-like repeats of unknown funtion; and an extracellular stretch of multivalent 27 CUB domains, representing nearly 85% of the total mass of the protein, and involved in binding to many protein ligands (see text) **(fig003bsw)**.

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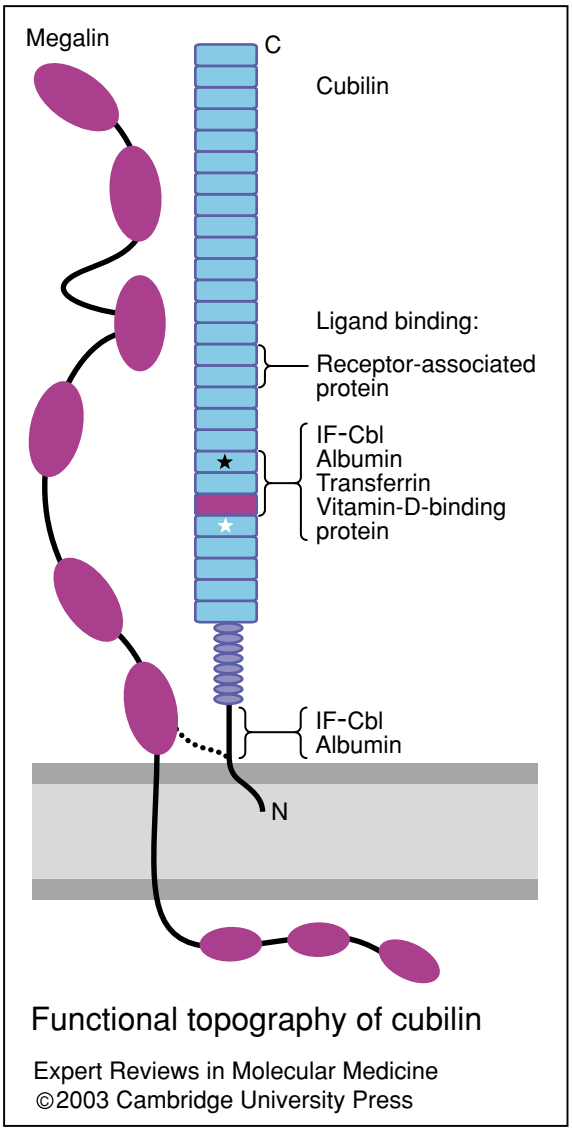

Figure 4. Functional topography of cubilin. A proposed model for the functional orientation of cubilin and megalin in the apical membranes of polarised epithelia. Cubilin is shown associating (dashed line) with the transmembrane protein megalin via the N-terminal domain of cubilin (cubilin itself lacks a transmembrane domain, but might interact with the membrane via palmitoylation). Several ligands are known to interact with the CUB domains, and in some cases binding regions on cubilin have been identified: CUB domains 6–8 are important in binding of IF (intrinsic factor)–Cbl, albumin, transferrin and vitamin D-binding protein; CUB domains 13–14 are important in binding to receptor-associated protein. The locations of alternative sites for IF–Cbl and albumin at the N-terminus are also shown. The sites of two disease mutations (FM1 in CUB8 and FM2 in CUB5) identified in Finnish patients are indicated by stars **(fig004bsw)**.

is thought to have other functions, such as aiding in the trimerisation of the cubilin molecule (Ref. 68) and interacting with megalin (Ref. 38), both of which could be important for making peripheral contact with the lipid bilayer (Fig. 4). Indeed, despite its role in the endocytosis of the many ligands it binds, cubilin itself has no cytoplasmic or transmembrane domains (Ref. 2). The structural elements of the N-terminal region of cubilin that make contact with the lipid bilayer are not known, but palmitoylation in this region might be involved as cubilin from opossum kidney cells has been shown to be palmitoylated (Ref. 12). On the basis of the in vitro binding of cubilin to the 600 kDa endocytic receptor megalin (Ref. 2), it is suggested that both endocytosis of the many ligands that bind to cubilin and its own membrane recycling are assisted by megalin (Ref. 2). In addition, megalin is thought to be a chaperone for the plasma membrane delivery of cubilin (Ref. 69).

In vivo evidence for the importance of the megalin–cubilin interaction has been obtained recently in whole-body-irradiated rats, which developed severe albuminuria due to loss of albumin and megalin but did not lose IF–Cbl binding to cubilin (Ref. 70). These observations support the distinct nature of albumin- and IF–Cbl-binding sites on cubilin and might help explain why some patients with IG syndrome exhibit proteinuria (i.e. both binding sites dysfunctional) whereas others do not (only the IF–Cbl-binding site dysfunctional). Although structural details of the cubilin–megalin interaction are not known, the N-terminal region of cubilin, including the eight EGF domains, has been implicated as one of the regions important in cubilin binding to megalin (Ref. 38). Loss of this region as a result of increased proteolytic conversion of the 460 kDa form of cubilin in the intestine of thyroidectomised rats resulted in decreased association of cubilin with megalin, as well as decreased absorption and transport of orally administered Cbl (Ref. 38). These studies have underscored the importance of cubilin–megalin interactions in the apical brush borders of kidney cortex and intestine but additional studies are required to identify other regions of cubilin that may interact with megalin. Such studies will help increase understanding of how megalin assists in influencing multiple ligand binding, internalisation and membrane recycling of cubilin.

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Additional roles for cubilin

Cubilin is an important molecule required for renal tubular reabsorption of many filtered proteins, vitamins and other ligands yet to be identified. In the intestine, however, it is not known whether cubilin has any function other than Cbl absorption. However, as a result of its ability to bind to galectin 3 (Ref. 71), a protein that plays a role in cell adhesion and tumour progression (Ref. 72), intestinal cubilin might play a role in regulating gastrointestinal cancers (Ref. 73) and in inflammatory responses (Ref. 74). Cubilin is also expressed in the yolk sac (Ref. 75) and could play an important role in the endocytic mechanisms that are important in nutrient transport at the maternal–foetal interface. Cubilin is generally found in the perforin-containing granules of the uterine natural killer cells and is endocytosed via galectin 3 (Ref. 71). Thus, cubilin–galectin-3 interactions might be important in uterine natural killer cell function and in the development of the foetus. Further investigations into the structure–function relationship of cubilin are required to understand how, by binding to different types of ligands, cubilin might affect intestinal and renal function and regulate foetal development.

The TC II receptor

Synthesis, membrane expression and interactions

TC II-R is initially synthesised as a 45 kDa protein that is then heavily glycosylated to form a mature protein of molecular mass 62 kDa; however, following its expression in the plasma membrane, it forms a functional noncovalent homodimer of molecular mass 124 kDa (Ref. 5). Dimerisation of TC II-R is driven by membrane fluidity; it occurs only in plasma membranes where the phospholipid:cholesterol ratio is 10:1 and cholesterol–phospholipid interactions are essential to keep the dimer intact (Refs 76, 77). TC II-R dimer levels represent nearly 80–90% of total tissue TC II-R protein levels in rat tissues tested, suggesting that at steady state most of the TC II synthesised is delivered to the plasma membranes (Ref. 78). Mature TC II-R contains four intramolecular disulphide bonds that are essential for its ligand binding, but not for its intracellular stability or its trafficking from the endoplasmic reticulum to the trans-Golgi (Ref. 79).

In addition to binding to TC II-R, TC II–Cbl also binds to megalin on renal tubular epithelial cells (Ref. 80). The binding appears to contribute to tubular reabsorption of filtered TC II, as megalin-knockout mice have elevated levels of urinary TC II (Ref. 81). However, more-recent studies (Ref. 82) have shown that megalin binds TC II-R with higher affinity than it binds TC II–Cbl. Thus, it is unlikely that megalin has a direct role in the tubular reabsorption of filtered TC II or in the plasma delivery of TC II–Cbl to cells with very little or no expression of megalin.

Endocytosis of TC II–Cbl in polarised epithelial cells

In human kidney and intestinal mucosa, TC II-R activity and protein levels are distributed between the apical and the basolateral membranes in the ratio of 1:6 and, in polarised intestinal epithelial Caco-2 cells, TC II-R is able to mediate endocytosis of TC II–Cbl from both plasma membrane domains (Ref. 83). However, apically endocytosed TC II–Cbl is transcytosed intact to the basolateral side whereas basolaterally internalised TC II is degraded in the lysosomes and Cbl is retained and utilised by the cells. Since orally administered labelled TC II–Cbl also crosses the mucosal barrier in rats (Ref. 83), it might be possible to deliver Cbl bound to TC II in patients who have undergone surgery of either stomach or terminal ileum or who are unable to synthesise TC II.

Regulation of expression

Several earlier studies (reviewed in Ref. 47 and citations therein) have shown by cell-surface TC II–Cbl binding that cell-surface TC II-R activity levels are upregulated to import more Cbl when the cells are in a proliferating mode. Available evidence using cultured lymphocytes (Ref. 84) and methionine-dependent P-60 human glioma cells (Ref. 85) have shown that cell-surface TC II-R levels are regulated by intracellular methyl-Cbl levels. When cultured in the presence of homocysteine, an immediate precursor of methionine, methionine-dependent P-60 human glioma cells showed a sixfold and twofold increase in TC II-R activity and intracellular Cbl levels, respectively. These studies have indicated that upregulation of TC II-R might occur in cells when intracellular methyl-Cbl demand increases, to meet increased demand for remethylation of imported homocysteine to methionine. This observation is interesting as it suggests that upregulation of TC II-R on the cell surface might

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be a property of tumour cells, which are highly proliferative and also dependent on methionine.

Several more-recent studies have shown that cell-surface TC II-R expression is indeed upregulated in cancer cells. These studies have demonstrated: (1) the ability of a monoclonal antibody against TC II that blocks its binding to TC II-R to inhibit proliferation of leukaemic cells (Ref. 86); (2) the ability to detect small tumours in rats (Ref. 87) and humans (Ref. 88) by whole-body radioimaging using a radiolabelled Cbl analogue that was imported in higher amounts to these sites in the body; and (3) a relationship between higher import of radiolabelled Cbl and increased TC II-R protein expression (Ref. 89) in ovarian cancer NIH-OVCAR-5 cells and tumour xenografts that were obtained using NIH-OVCAR-5 cells in nude mice.

Interestingly, higher TC II-R levels noted in NIH-OVCAR-3 cells required interferon β treatment (Ref. 89), a characteristic of a family of cell-surface markers containing Ig domains, such as human leukocyte antigens HLA-I and HLA-II, β_2-microglobulin and carcinoembryonic antigen. This raises the possibility that TC II-R also belongs to this family of proteins and contains an Ig domain. In support of this suggestion are recent observations that have shown the binding of pure human TC II-R to bacterial IgG-binding proteins such as *Staphylococcus aureus* protein A (Ref. 90). Moreover, radiolabelled Cbl uptake in human cells was inhibited by both antiserum to the Ig Fc region and by protein A. Taken together, these observations have indicated strongly that human TC II-R is an IgG-like protein. However, further studies are required to understand the structural elements of TC II-R and the role of IgG-like domains in TC II binding and internalisation.

Developmentally, TC II-mediated uptake of Cbl has been shown to occur in germ cells (Ref. 91) and murine yolk sac (Ref. 92), and immunoblot studies using rat tissue membranes from rats of different ages have shown that TC II-R protein levels are not temporally regulated during postnatal development (Ref. 78) – although their tissue levels vary, being highest in the kidney. TC II-R protein and activity levels and plasma transport of absorbed Cbl to peripheral tissues are regulated by the hormone cortisone, as all three were drastically reduced in adult adrenalectomised rats (Ref. 78), an effect that was completely reversed following cortisone treatment of these animals. The physiological relevance, if any, of prenatal expression of TC II-R in Cbl transport to developing foetal tissues is not known, and further studies are needed to understand whether cortisone exerts its effect at transcriptional or translational levels.

Concluding remarks

Rapid strides have been made in the past ten years in understanding the molecular structure of Cbl-binding proteins and their cell-surface receptors. These include the cloning of genes encoding IF and TC II, establishing the molecular basis of human TC II deficiency, elucidating the structure and many functions of cubilin, and identifying two receptors for TC II and the functional importance of normal TC II-R (Ref. 93). Another important dimension, particularly of cubilin and TC II-R, is their interaction with megalin and the ability of cubilin to interact with galectin 3, an animal lectin important in cancer and inflammation. Further studies in this area should focus on the structural analysis of these proteins, particularly cubilin and TC II-R, and the effects of their gene disruption in health and disease.

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http://www.emedicine.com/med/topic1799.htm
http://www.mssc.edu/biology/B305/GTS/fs99/anemia/anemia.htm

Features associated with this article

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Tables

Table 1. Inherited disorders of cobalamin (Cbl; vitamin B_{12}) malabsorption (tab001bsw)
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