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Advances in the Understanding of Cobalamin Assimilation and Metabolism

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Summary

The haematological and neurological consequences of cobalamin deficiency define the essential role of this vitamin in key metabolic reactions. The identification of cubilin-amnionless as the receptors for intestinal absorption of intrinsic factor-bound cobalamin and the plasma membrane receptor for cellular uptake of transcobalamin bound cobalamin have provided a clearer understanding of the absorption and cellular uptake of this vitamin. As the genes involved in the intracellular processing of cobalamins and genetic defects of these pathways are identified, the metabolic disposition of cobalamins and the proteins involved are being recognized. The synthesis of methylcobalamin and 5'deoxyadenosylcobalamin, their utilization in conjunction with methionine synthase and methylmalonylCoA mutase, respectively, and the metabolic consequences of defects in these pathways could provide insights into the clinical presentation of cobalamin deficiency.

Keywords

Vitamin B12; Transcobalamin-receptor/CD320; Intrinsic factor; haptocorrin

1. Introduction

During the eighty plus years since the discovery of vitamin B_{12} (cobalamin, Cbl), much has been written to define the clinical, haematological and neuropathological manifestations of the disorders that result from Cbl deficiency (Watkins *et al*, 2009). Even though the haematological presentation of Cbl deficiency cannot be distinguished from that of folate deficiency (Wickramasinghe, 2006), the clinical disorder has, to a large extent, been defined as presenting with neuropathological changes that are unique to Cbl deficiency (Healton *et al*, 1991). However, this assumption may be changing due to the association of folate deficiency with gross structural alterations of neural development during embryonic and fetal life (Finnell *et al*, 2008) and functional abnormalities observed in children with cerebral folate deficiency syndrome (Ramaekers & Blau, 2004). The neurological presentation of dementia and diminishing cognitive function in the older population may well result from the combined deficiency of Cbl and folate in the central nervous system (Reynolds, 2006) however, definitive proof of this hypothesis is lacking. Recent advances in the purification of proteins and molecular genetics have provided new and definitive information on the Cbl binding proteins, their receptors, and enzymes involved in the intracellular processing of Cbl. This fundamental information has also enabled us to identify gene defects involving pathways of absorption, cellular uptake and intracellular metabolism of Cbl (Figure 1).

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2. Cobalamin homeostasis

In recent years, there has been much debate on Cbl status and subclinical Cbl deficiency. Approximately 6% of the western population over the age of 60 years has low plasma Cbl and as much as 20% may have marginal Cbl status (Allen, 2009). This subtle deficiency may play a role in decreased cognitive function and dementia in the ageing population (Smith & Refsum, 2009). Decreased dietary intake and poor absorption may contribute to low Cbl status in this population (Lindenbaum *et al*, 1994). The daily intake of Cbl in a western diet is probably greater than 5μg. This is likely to be considerably less in eastern and vegetarian diets due to lower meat intake. Based on daily loss of 1 – 2 μg of Cbl, a similar amount will have to be absorbed to maintain normal Cbl status. In Cbl deficiency, body stores would probably deplete in 6 – 12 months based on a half-life of about 12 months and loss of about 2 –4 μg/day because of impaired reabsorption (Chanarin, 1979). The folate supplementation of diet to reduce the incidence of neural tube defect pregnancy and to lower homocysteine (HCY) to decrease the risk of cardio-vascular disease (Selhub & Rosenberg, 2008) has raised issues about the need to improve the Cbl level along with the folate (Thompson et al, 2009; Green, 2009). Therefore, the current perception of normal plasma Cbl in the 200 – 800 pg / ml range, may need re-evaluation and a higher daily intake of Cbl may have to be considered. This may be particularly relevant in the elderly population with decreased absorption of dietary Cbl (Carmel, 1997). Given that approximately 80% of plasma Cbl is bound to haptocorrin (HC) and because it is the fraction bound to transcobalamin (TC) that is available for cellular uptake, a daily higher intake would provide more Cbl for tissue cells. Even though including Cbl in food fortification has been debated, universal agreement on this issue has not been reached. One potential objection has been the issue of stability of Cbl and the likelihood of introducing analogs or breakdown products that may be harmful antimetabolites. A resolution to this dilemma may be to fortify a food source, such as milk, with Cbl. With a short shelf life and storage under refrigerated conditions, and because some quantity is consumed in various forms by most of the population, milk could provide a vehicle to increase the daily intake to raise the Cbl status in the population. The percentage of Cbl absorbed decreases with increasing dose and less than 2μg of a 50μg dose is absorbed. This may be considered as the limit of Cbl absorption via the intrinsic factor (IF) receptor pathway since the amount of IF produced is far in excess of the amount of Cbl absorbed. At doses of 1–2mg, approximately 10μg of the vitamin may be absorbed via nonspecific internalization in both normal subjects and in patients with malabsorption (Carmel, 2008). Such high doses of oral Cbl are considered as effective as periodic intramuscular injections in restoring Cbl status (Butler *et al*, 2006).

3. Absorption of Cobalamin

The process of dietary Cbl absorption has evolved to provide selective absorption of Cbl and exclude structurally similar compounds of bacterial origin that may come from gut microflora and dietary sources. This process is mediated by a number of proteins, haptocorrin (HC) in the saliva, intrinsic factor (IF) in gastric juice and the receptor for IF-Cbl in the distal ileum.

Intrinsic Factor

This protein is produced by the acid secreting pareital cells of the stomach and binds ~50–60 ng Cbl per ml of gastric juice. Based on an average gastric juice production of 1–3 litres / 24 h, approximately 1–5mg of IF is produced to bind about 30–150 ug of Cbl (Allen, 1975). Thus, available IF is far in excess of the daily amount of Cbl absorbed. The 45 kDa protein is glycosylated and sialydated, accounting for the 58–60 kDa size by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Gueant *et al*, 1985). The gene (*GIF*) encodes a 417 amino acid protein with 9 exons, spans a region of 16.2 kb and is

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located at11q13 (Hewitt *et al*, 1991). The recombinant IF produced in plant has been well characterized. Based on these data, the protein shows two distinct domains with each domain capable of binding Cbl. However, as a single protein, both domains appear to cooperatively participate in binding Cbl with higher affinity than each domain separately (Fedosov, *et al*, 2005a). The corrin ring sits parallel to the central axis of the alpha barrel and forms multiple hydrogen bonds with both domains. Among the various Cbl binding proteins, HC has the broadest affinity for Cbl analogs, whereas IF specifically binds Cbl and reacts poorly with Cbl analogs, such as cobinamide, which lacks the nucleotide portion. The specificity for Cbl and Cbl analogs appears to reside in the beta 3 and beta 4 regions of the beta domain. The difference in amino acids in these regions among the three Cbl binding proteins accounts for the difference in affinity (Wuerges *et al*, 2007; Mathews *et al*, 2007). The amino acids in this region of IF appear to be critical for the specificity of Cbl binding and may have evolved to ensure selective absorption of Cbl in an environment where corrin-like compounds of bacterial origin or from break down of food Cbl may compete for the limited absorptive capacity of the IF receptor. The specificity for Cbl, coupled with the fact that the complete 50 kDa protein is required for receptor recognition, implies that the tertiary structure of the 50 kDa IF with the Cbl in position is necessary to generate the appropriate conformation for its interaction with the receptor for IF-Cbl.

The existence of a receptor in the distal ileum for absorption of Cbl was demonstrated in early studies by Ca^{++} dependent binding of IF-Cbl. The definitive identification of the receptor protein proved to be difficult because of the multiple protein bands in the purified product. The difficulty in obtaining a homogeneous preparation of the receptor protein for conclusive identification may be attributed to the fact that the protein now identified as the receptor for IF-Cbl is a receptor for multiple ligands and interacts with at least two other membrane proteins (Moestrup & Verroust, 2001). Decreased absorption of Cbl can result from many causes, the most common of which is pernicious anemia (PA) due to autoimmune destruction of parietal cells (Chanarin, 1979). Although of adult onset, this disorder can present in children and young adults as juvenile PA. Inborn errors of IF are rare and range from a total lack of IF to a non-functional protein. The latter could present as decreased binding of Cbl or to the receptor or as an unstable protein (Reviewed in Rosenblatt & Fenton, 1999). The genetic defects contributing to these abnormalities were not identified. The first *GIF* defect identified was a four base deletion in exon 2 (c183 - 186 delCAAT) leading to a frame shift and premature termination of translation. This was described in a female child of African descent (Yassin *et al*, 2004). Three additional cases have been identified and the identical deletion in this population has been attributed to a founder effect (Tanner *et al*, 2005; Ament *et al*, 2009). This homozygous mutation appears to be due to autosomal recessive inheritance of the *GIF* defect. A number of other single mutations leading to loss of function and polymorphisms have been identified (Tanner *et al*, 2005). Polymorphism in exon 1 (Q5R) was reported in four patients with low IF (Gordon *et al*, 2004). However, a larger study has identified this polymorphism at a frequency of 3 – 11% and may not be associated with decreased IF production (Ament *et al*, 2009) or low Cbl (Remacha *et al*, 2008).

Intrinsic Factor Receptor and the absorption of Cbl

The absorption of physiological concentrations of Cbl occurs via IF and the receptor for IF-Cbl, cubilin (CUB), a peripheral membrane-associated protein expressed in the distal ileum. This 3600 aa, 460 kDa protein contains 8 epidermal growth factor (EGF)-like repeats followed by 27 repeats of 110 aa of CUB (complement components C1r/C1s, the sea urchin protein Uegf, and bone morphogenic protein-1) domains found in many developmentally regulated proteins and involved in ligand binding (Kozyraki & Gofflot 2007). The binding of IF-Cbl has been localized to CUB domains 5–8 (Kristiansen *et al*, 1999). The human gene

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(*CUBN*) is located at10p12.31 and spans a region of 306kb with 39 exons (Kozyraki *et al*, 1998). The internalization of CUB with IF-Cbl is facilitated by amnionless (AMN), a transmembrane protein originally identified as a visceral endoderm protein essential for embryonic development. Congenital malabsorption of Cbl can result from mutations in either protein because AMN appears to be involved in the apical targeting as well as internalization of CUB (Fyfe *et al*, 2004). Even though megalin (MAG) and receptor-associated protein (RAP) can interact with CUB, the precise role if any, of these proteins in CUB-mediated absorption of IF-Cbl has not been determined.

3. Transport of Cobalamin

The IF-Cbl absorbed in the distal ileum appears in the circulation bound to TC. The events following translocation of IF-Cbl to the ileal enterocyte, release from IF and binding to TC are not fully defined. However, based on *in vivo* and *in vitro* studies, it is evident that apo TC is available in the ileum to bind the free Cbl. Evidence in support of this conclusion is based on: 1. In human studies, orally administered radiolabeled Cbl appeared bound to TC (Chanarin *et al*, 1978); 2. IF-Cbl absorption studies in the guinea pig showed the appearance of TC-Cbl from the isolated ileum (Rothenberg *et al*, 1978). The synthesis of TC in the intestinal villous and its transport to the serosal side has been confirmed by in situ hybridization studies and in the isolated ileum (Quadros *et al*, 1999). The distribution of the mRNA and the appearance of apoTC in the villous would support the origin of TC in the abundant microvascular endothelium. The release of Cbl from IF and its exit from the ileal enterocyte is not well defined. The Cbl may exit the cell by simple or facilitated diffusion against a concentration gradient or a yet unidentified active transport system may operate to release the Cbl into the basolateral side. A transport system for free Cbl was proposed for the exit of Cbl from L-1210 cells cultured *in vitro* (Quadros and Jacobsen, 1995) and may operate in all cell types for disposition and recycling of Cbl.

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Cbl concentration in plasma has been used as a measure of nutrient status for decades and provided a reasonable parameter to distinguish normal from severe deficiency. However, the availability of assays for sensitive indicators of intracellular metabolites, such as methylmalonic acid (MMA), homocysteine (HCY) and 5-methyl citrate (Savage *et al*, 1994) have questioned the validity of total Cbl estimation, which does not always correlate with the clinical presentation of Cbl deficiency. Given that ~80% of the Cbl in serum is bound to HC and is not readily available for cellular uptake, the determination of Cbl specifically bound to TC (holotranscobalamin; holo-TC) was suggested as an alternative measure of Cbl status (Herzlich & Herbert, 1988). This would provide a measure of daily Cbl intake and Cbl readily available for cellular uptake because newly absorbed Cbl would be bound to TC. The availability of sensitive and direct assays for holo-TC (Brady *et al*, 2008) have overcome many of the technical limitations and recent studies provided convincing proof of the merits of assaying holo-TC (Morkbak *et al*, 2005). Even though this assay could replace total Cbl determination for mass screening, ultimately, intracellular metabolites, especially MMA, in conjunction with serum holo-TC may provide the most information on Cbl status and utilization.

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Transcobalamin (TC)

This nonglycosylated protein in plasma carries between 10–30 % of the total Cbl and is mostly present in its apo form (apotranscobalamin, apo-TC). Cohn fraction III of human plasma was the source of apo-TC for obtaining highly purified TC (Quadros, *et al*, 1986). TC serves the essential function of binding and transporting the newly absorbed Cbl in the distal ileum to tissue cells throughout the body where it is internalized by receptor-mediated cellular uptake (Rothenberg &Quadros, 1995). Even though all cells may produce some TC, the synthesis of large amounts of this protein by human umbilical vein endothelial cells

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suggests the endothelial cell as the likely source of this protein in circulation (Quadros *et al*, 1989). The abundant vascular endothelium is an ideal source of this protein with a relatively short half-life of 1–2 h for holo-TC and rapid uptake by tissue cells. This major source of TC also facilitated the cloning of the cDNA from an endothelial cDNA library (Platica *et al*, 1991). The gene encoding TC (*TCN2*) is located at 22 q11-13.1, spans a region of 18kb and has 9 exons. The similarity in the organization of the genes and amino acid sequences encoding Cbl binding proteins suggests their origin from a common ancestral gene (Regec *et al* 1995). The 1.9 kb cDNA encodes a peptide of 409 aa, 45.5 kDa secreted protein. Single nucleotide substitutions were identified at codon 198, 219, 259 and 376 (Li *et al*, 1993) however, the two isoforms of TC observed are due to the difference in two amino acids in the N terminus of the secreted protein (Quadros *et al*, 1993). TC is more discriminating than HC in binding Cbl analogues. Within the corrin ring, the amide side chains appear to be critical for binding, with modification of the amide group at the "e" position having the least effect (Pathare *et al*, 1996). Modifications or addition of ligands to the central cobalt or to the ribose moiety of the nucleotide base does not affect binding to TC. The crystallographic analysis of bovine holo-TC has provided the structure of TC and Cbl binding (Wuerges *et al*, 2006). The amino terminal major domain and the carboxy terminal minor domain appear to form a pocket for the Cbl molecule. It is the folded conformation of TC with the Cbl embedded that provides the epitope(s) for interaction with the receptor (TCblR). Based on the deduced structure and epitope mapping of the TC protein with monoclonal antibodies (Quadros *et al*, 1996), residues 103–159 and the positively charged heparin-binding region in residues 207 – 227 are likely to be involved in receptor binding (Fedosov *et al*, 2005b).

Genetic abnormalities of Transcobalamin

Congenital TC deficiency is not embryonically lethal and infants with this defect are born normal. However, these infants develop Cbl deficiency soon after birth and therefore prompt diagnosis and treatment with pharmacological doses of Cbl are critical to preventing hematological and neurological complications. Congenital abnormalities of TC include complete absence of TC, immunoreactive TC that does not bind to the receptor or that does not bind Cbl (reviewed in Rothenberg & Quadros, 1995). Mutations in *TCN2* leading to TC deficiency have been identified and these can be deletions, nonsense mutations (Li *et al*, 1994), errors in RNA editing of the primary transcript (Qian *et al*, 2002) and a point mutation in the intron 3 splice site, generating a cryptic splice site in exon 3 and an in-frame deletion of 81 nucleotides (Namour *et al*, 2003).

Haptocorrins (HC)

The proteins referred to in older literature as R-binder, TC1 or TCIII are collectively named haptocorrins and differ only in glycosylation (Alpers & Russell-Jones, 1999). They are the product of a single gene (*TCN1*) encoded by 9 exons on chromosome11 and are present in many body fluids and secreted by many cell types including glandular cells (Johnston *et al*, 1989). The 410 aa core polypeptide accounts for a molecular weight of 45.5 kDa and the 30–40% glycosylation accounts for the ~66 kDa size (Burger *et al*, 1975). The desialylated protein can be taken up and degraded by the asialoglycoprotein receptor in the liver (Alpers & Russell-Jones 1999). No specific function has been assigned to this protein other than binding a large fraction of Cbl in circulation. This may prevent loss of free Cbl and its lack of specificity for Cbl may aid in clearing corrin–like compounds from entering the cell via the TC-receptor pathway.

4. Cellular uptake of Cbl

Early studies had recognized that free Cbl is not internalized in cells and a serum component is required for cellular uptake of physiological concentrations of Cbl. The serum component

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was subsequently identified as TC. Studies to define the pathway for cellular uptake of Cbl provided clear evidence of enhanced uptake of TC bound Cbl. This uptake involved binding of TC-Cbl to the cell surface and internalization of the TC-Cbl complex. The process required the divalent cation Ca^{++} and metabolic energy as indicated by enhanced uptake at 37°C (DiGirolamo & Huennekens, 1975). Even though the existence of a receptor on the plasma membrane for cellular uptake of TC-Cbl could be readily inferred from binding and uptake studies in live cells, the purification of the receptor protein proved to be a formidable challenge to investigators. However, functional receptor could be demonstrated by binding of TC-Cbl to a detergent soluble fraction of placental membranes analyzed by gel filtration chromatographic separation of the receptor/TC-Cbl complex (Nexo & Hollenberg, 1980).

The Receptor for Transcobalamin-Cbl

The very first concerted attempt to purify the soluble receptor used human placental membranes as the source of the receptor and affinity chromatography on a TC-Cbl matrix as the main purification step (Seligman & Allen 1978). The final product was, at best, partially pure and therefore, the data obtained on the size of the protein and amino acid/carbohydrate composition cannot be considered as accurate. Our initial attempts to purify the receptor from human placenta proved difficult. However, we were able to obtain information on the binding kinetics of the membrane bound and soluble receptor and on the size of the protein by cross-linking experiments and identified a ~58 kDa protein that was extensively glycosylated (Quadros *et al*, 1994). Following our publication, purification of the receptor was reported by another group that identified a 72 kDa monomer / 144 kDa dimer as the receptor on the plasma membrane for TC-Cbl (Bose *et al*, 1995). The reported abundance of this protein in placenta and the lack of any confirmation of specific binding to TC-Cbl, convinced us that the protein described with an apparent FC-like domain (Vanamala *et al*, 2003) may not be the receptor for TC-Cbl. We experimentally proved that the protein solubilized from human placental membrane that specifically binds TC-Cbl with high affinity does not have a FC-like domain (Quadros *et al*, 2005). This was followed by about two years of studies using refinements in conventional protein purification, coupled with multiple affinity purification steps that ultimately provided what appeared to be a homogeneous single protein by SDS-PAGE. The 58 kDa size of the protein and its retention through multiple affinity purification steps in association with TC-Cbl convinced us that the protein was the receptor for TC-Cbl. The receptor protein (TCblR) was identified by liquid chromatography / mass spectroscopy analysis of the trypsin-digested peptide fragments and the gene (*CD320*) encoding this receptor was identified from the human genome data bank (Quadros *et al*, 2009), Structurally this 282 aa protein belongs to the LDL receptor family with two LDLR type A domains separated by a 55 aa cysteine rich CUB-like domain, a 21 aa transmembrane region and a 32 aa cytoplasmic domain. There are three N glycosylation sites in the 198 aa extracellular region with potential O glycosylation sites both in the extracellular as well as cytoplasmic domains. The aberrant migration in SDS-PAGE as a 58 kDa protein is probably due to extensive glycosylation of the protein. The two LDLR type A domains and the intervening cysteine-rich CUB-like domain show remarkable similarity to other receptors of this family of proteins. However, the small size, the presence of only two LDLR-A domains and the sequences unique to these two domains probably provide the specificity for TC-Cbl binding because LDL and RAP, the two proteins known to affect binding of ligands to this class of receptors, do not affect TC-Cbl binding.

The expression of TCblR on the cell surface is fairly low with maximum expression in actively dividing cells and down-regulation of functional receptors in quiescent cells (Lindemans *et al*, 1989; Amagasaki *et al*, 1990). This cell cycle association of receptor expression and the efflux of free Cbl out of the cell may be physiologically relevant in that receptors are expressed to meet increased Cbl requirement during DNA synthesis and once

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this requirement has been met, the excess Cbl is effectively transported out of the cell and is captured by circulating apo-TC for mobilization to where it is needed most. Studies using *in vitro* culture of cells have supported the notion that the receptor is cell cycle associated, the TC-Cbl is internalized by endocytosis via clathrin-coated pits, the TC-Cbl is degraded in the lysosome and the receptor is recycled to the plasma membrane in a manner similar to the transferrin receptor (Takahashi *et al*, 1980). These conclusions need confirmation and with the identification of the receptor protein and available tools, such as epitope specific antibodies and receptor tagged with fluorescent proteins, it is now possible to study in detail the process of TC-Cbl binding, internalization and fate of the receptor.

Genetic abnormalities of *CD320* have not been reported. However, the recent identification of the gene provides the opportunity to investigate mutations of this essential receptor contributing to decreased cellular uptake of Cbl. Newborns identified with elevated MMA and HCY that cannot be attributed to known causes of defects in Cbl metabolism should be investigated for potential defects in *CD320* contributing to decreased cellular uptake of Cbl.

Embryonic uptake of Cbl

The observation of higher holo-HC and TC levels in cord blood than in maternal blood suggests increased demand and turnover of Cbl in the fetus (Obeid *et al*, 2006). In this case, maternal Cbl status is an important determinant of transplacental transport of Cbl to meet the fetal Cbl requirement. In congenital TC deficiency, the fetus is not Cbl-deficient and develops normally to full term. In this case, maternal TC must provide the required Cbl. Our recent work on the receptor for TC-Cbl has indicated that the receptor gene knockout is not lethal to the embryo (unpublished data). Therefore, a surrogate TC receptor must function in the developing embryo to provide the necessary Cbl. IF and HC are present in amniotic fluid (Aimone-Gastin *et al*, 1999) and both CUB and MAG are expressed very early, during 8–16 cell stage of the embryo (Assemat *et al*, 2005), and therefore could serve as ligands and receptors for Cbl uptake. Gene knockout or antibodies to CUB and MAG are lethal and show multiple malformations. These abnormalities cannot be attributed to specific nutrients because of the diverse role of these receptors in internalizing numerous ligands (Kozyraki & Gofflot 2007). Increases in Cbl binders and decreases in total Cbl observed in mothers during pregnancy and at term, may be related to hormonal changes during pregnancy (Fernandes-Costa & Metz 1982). Follow up studies of women from preconception to delivery indicates progressive decrease in total and holo-TC and an increase in MMA in the cord blood at the time of delivery (Murphy *et al*, 2007). Increased Cbl requirement and perhaps up regulation of propionate metabolism may account for this. During early infancy, the Cbl requirement is met by breast milk, the only source of nutrition during this period, because a substantial portion of the Cbl intake of the mother is transferred to breast milk (Linnell, 1975).

The liver and kidney in cobalamin homeostasis

The primary storage sites for Cbl are the liver and kidney. The liver contains ~10 μg Cbl/g protein or 1 μg/g wet weight and can carry between 1–1.5 mg of the vitamin. The stored amount increases with age from newborn to adult (Rappazzo *et al* 1970). As the bulk of the liver mass is due to hepatocytes, HC-bound Cbl taken up via the asialoglycoprotein receptor could account for most of the stored Cbl (Alpers & Russel-Jones, 1999). Similarly, in the kidney, it would be due to renal reabsorption of TC-Cbl by megalin (Moestrup *et al*, 1996). The precise mechanism by which this storage occurs is not clear. As very little Cbl is distributed as free Cbl in the tissue, the assumption is that it is mostly bound to the two enzymes, methionine synthase (MS) and methylmalonylCoA Mutase (MMU). However, both enzymes are only partially saturated with Cbl even under Cbl replete conditions (Kolhouse *et al*, 1980) and no direct correlation between the activity of these enzymes and

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Cbl-replete status exists. The mechanism of Cbl release from its storage site under Cbl deficient conditions is also not known. Typically, the fall in serum Cbl precedes the fall in liver stores (Booth & Spray 1960). Based on the *in vitro* cell culture model (Quadros & Jacobsen, 1995) and tracer studies in animal models (Linnell 1975), it appears that the uptake, utilization and release of Cbl is a fairly dynamic process, with the Cbl and the various proteins associated with its utilization in constant flux. The accumulation of Cbl in some tissues like liver and kidney appears to be the exception that cannot be explained by the dynamic flux and raises a number of questions. For example, what factors regulate the accumulation of Cbl in liver and kidney? How does the Cbl-replete or Cbl-deficient status affect this process and the two enzymes? Does the stored Cbl compartmentalize to a location other than the two enzymes? Does the Cbl status affect synthesis, turnover and saturation of Cbl-dependent enzymes?

5. Metabolic utilization of Cbl and Inborn errors

Our current understanding of intracellular Cbl interconcersion indicates that once Cbl is released from TC, it undergoes processing to coenzyme forms and is directed to the two Cbl-dependent enzymes, MS and MMU. The intracellular distribution of various forms of Cbl has been well documented and in all tissues 5’ deoxyadenosyl Cbl (AdoCbl) is the predominant form with lesser amounts of hydroxo-Cbl. Methyl Cbl (MeCbl) is a minor component of intracellular Cbl. However, MeCbl is the major form of Cbl in the plasma and it is this form that is disproportionately reduced in Cbl deficiency. Higher MeCbl levels were observed in fetal tissues in association with higher MS enzyme activity (Linnell, 1975). A similar association was observed with cells cultured in medium containing methyl folate and HCY that resulted in a 3-fold increase in the levels of both holo- and apo-MS and a 9-fold increase in MeCbl, suggesting that the synthesis of MeCbl was tied to the activity and turnover of MS enzyme (Quadros & Jacobsen, 1995). The identification of methionine synthase reductase (MSR) enzyme (Leclerc *et al*, 1999) and the current understanding of the action of MSR in conjunction of MS enzyme activity (Wolthers & Scrutton 2009), provides compelling proof that MeCbl synthesis is linked to the catalytic activity of MS and the level of MeCbl in the plasma is a direct reflection of effective functioning of this pathway. The initial steps in the synthesis of Cbl coenzymes appear to be in the efficient removal of the upper axial ligand attached to the central cobalt, irrespective of the form of Cbl transported into the cell as shown by efficient and rapid interconversion of labeled cyano-Cbl, AdoCbl and MeCbl (Quadros *et al*, 1979). This function was ascribed to Cbl reductases (Watanabe & Nakano 1997) involved in the stepwise reduction of trivalent cobalt to the divalent and then to the monovalent state and in the process, removing the upper axial ligand for the chaperoning of the Cbl to cytosolic MS or mitochondrial MMU where it would be converted to the respective coenzyme form (Banerjee, 2006). A recent report has identified a gene product of *MMACHC* (CblC) as the protein specifically involved in decyanation as well as dealkylation of Cbl (Hannibal *et al*, 2009). Therefore, MMACHC protein probably has broad specificity for removing all upper axial ligands in the neosynthesis of intracellular AdoCbl and MeCbl. A system for efficient reduction of cobalt in Cbl and interconversion of all forms of Cbl probably involves multiple enzymes and cofactors. The ATP-dependent reductive alkylation of the cobalt in Cbl has been specifically assigned to the mitochondria, however, occurrence of this in the cytoplasm can not be ruled out.

The genetic defects of Cbl metabolism specifically involve the intracellular processing and utilization of Cbl and include lysosomal release of free Cbl and enzymes involved in the synthesis and utilization of Cbl cofactors (Table 1). Disorders involving the synthesis of Cbl cofactors are identified as cblA to cbl G based primarily on the order in which they were discovered. Both cblA and cblB disorders involve defects in mitochondrial synthesis of AdoCbl. The two disorders differ in that cell extracts from cblA mutants can convert cyano

Cbl to AdoCbl whereas cblB mutants lack this ability (Whitehead, 2006). *In vivo*, a step common to these processes, such as transport of Cbl into mitochondria and / or initial steps in reductive alkylation, may be defective. The defect in $cblA^{-}$ has been ascribed to the *MMAA* gene (Dobson *et al*, 2002a) whose function is yet to be confirmed and that of $cblB^{-}$, to the *MMAB* gene, which encodes the enzyme for transfer of the adenosyl moiety from ATP to form AdoCbl. (Dobson *et al*, 2002b). The cblC, cblD and cblF phenotypes affect the synthesis of both MeCbl and AdoCbl and present with homocysteineuria and methylmalonic aciduria (Morel *et al*, 2005). These disorders are not fully characterized, however, the defect in cblF phenotype appears to be in the lysosomal exit of Cbl due to a defect in *LMBRD1* (Rutsch *et al*, 2009). Mutations in *MMACHC* result in the cblC phenotype (Lerner-Ellis *et al*, 2006). *MMADHC* carrying the cblD genotype results in elevated MMA and HCY. Mutations in exons 3 – 4 have been associated with MMA and with HCY in exons 6–8. In the case of combined MMA and HCY, additional mutation in exon 5 has been identified. Based on the phenotypes of the disorder, two functional domains have been suggested for this protein (Coehlo *et al*, 2008). The cblE and cblG defects are due to decreased activity of MS. The cblG disorder is due to mutations in the gene encoding MS (*MTR*) while the cblE disorder is due to mutations in *MTRR*, which encodes MSR associated with MS and is involved in the activation of MS and conversion of enzyme bound Cbl to MeCbl (Leclerc *et al*, 1999). One of the earliest genetic abnormalities of Cbl identified was in infants with MMA due to defects in the MMU enzyme. The gene defect can present as partial (MUT^{-}) or complete loss (MUT^{o}) of enzyme activity (Ledley & Rosenblatt, 1997).

Targeting the cobalamin pathways in cancers

The successful use of inhibitors of dihydrofolate reductase and thymidylate synthatase proved the utility of inhibiting folate pathways in cancer therapy. Since the identification of the role of Cbl-dependent MS enzyme in the recycling of folate, the use of Cbl antimetabolites as inhibitors of MS has been suggested as a potential strategy for cancer therapy (Huennekens *et al*, 1976). Even though numerous compounds were produced and tested none proved effective inhibitors of MS- or Cbl-dependent pathways. Newer approaches have explored the use of fluorescent Cbl compounds to visualize tumours, Cbl-drug conjugates to deliver these compounds preferentially to tumor cells (Hogenkamp *et al*, 1999), and Cbl depletion as a strategy to inhibit the proliferation of cancer cells (McLean *et al*, 1997). Using epitope-specific monoclonal antibodies to TC and TCblR, it is possible to block Cbl uptake or deliver Cbl drug conjugates via this pathway (Quadros *et al*, 1996). The increased Cbl requirement in highly proliferative cancer cells and the over expression of TCblR to meet the higher demand for Cbl, renders the TC-TCblR pathway an ideal target for this approach. Elevated haptocorrin has been reported in hepatocellular carcinomas and myelomas. This protein provides a marker for these malignancies and could serve as a vehicle for targeted therapy of tumors that express high levels of HC and receptors for this protein (Waibel et al 2008)

Epilogue

Over a century of research into the understanding of the pathology and metabolism of Cbl has resulted in our current understanding of the physiology and biochemistry of this essential vitamin and the transport proteins, receptors and genes involved in this process. The metabolic utilization of Cbl in the cell and the proteins involved is being unravelled; aided primarily by our understanding of inborn errors in these pathways. As a more complete picture of Cbl homeostasis emerges, strategies for effective prevention and treatment of Cbl deficiency disorders will be implemented.

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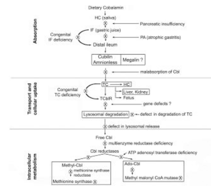

Figure 1.

Pathways and proteins involved in the assimilation of cobalamin and inborn or acquired defects (⊗) in these pathways.

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Table 1

Inborn Errors of Intracellular Cobalamin Metabolism

Disorder	*Gene involved	Chromosomal location Size/Exons	Protein involved	Disease phenotype	Biochemical abnormality
cblA	*MMAA*	4q31.21 39kb/6ex	46.5 kDa 418 aa	AdoCbl deficiency in cells/AdoCbl formation in cell free extracts	Methylmalonic aciduria
cblB	*MMAB*	12q24.11 19.8kb/9ex	27.4 kDa 250 aa	No AdoCbl formation in cells or in cell extracts	Methylmalonic aciduria
cblC	*MMACHC*	1p34.1 10.8kb/5ex	31.7 kDa 282 aa	Lack of AdoCbl and MeCbl synthesis	Homocysteinuria Methylmalonic aciduria
cblD cblD1	*MMADHC*	2q23.2 18kb/7ex	32.8 kDa 296 aa	Lack of AdoCbl and MeCbl synthesis 1No MeCbl, normal AdoCbl	Homocysteinuria Methylmalonic aciduria
cblE	*MTRR*	5q15.2 32kb/14ex	77.7 kDa 698 aa	Methionine synthase reductase deficiency	Low MeCbl Low MS activity Elevated HCY
cblF	*LMBRD1*	6q13 121kb/31ex	61.4 kDa 540 aa	Defective lysosomal exit	Lack of MeCbl and AdoCbl
cblG	*MTR*	1q42.3 1400.3kb/31ex	140 kDa 1265 aa	Decreased methionine synthase	Homocysteinuria Lack of MeCbl
MUT ^{o} MUT $^{-}$	*MUT*	6p12.3 30kb/13ex	83 kDa 750 aa	^{o}Lack MUT protein $^{-}$Partial MUT activity	Methylmalonic aciduria

* *MMAA*: methylmalonic aciduria cblA type

MMAB: methylmalonic aciduria cblB type

MMACHC: methylmalonic aciduria cblC type with homocysteinuria

MMADHC: methylmalonic aciduria cblD type with homocysteinuria

MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase/methionine synthase reductase.

LMBRD1: Initially labeled as limb regeneration domain 1(Identified as a lysosomal exporter)

MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase/ methionine synthase

MUT: methylmalonyl Coenzyme A mutase