Processing of alkylcobalamins in mammalian cells: A role for the MMACHC (cblC) gene product

Luciana Hannibal, Jihoe Kim, Nicola E. Brasch, Sihe Wang, David S. Rosenblatt, Ruma Banerjee, Donald W. Jacobsen

Abstract

The MMACHC gene product of the cblC complementation group, referred to as the cblC protein, catalyzes the in vitro and in vivo decylation of cyanocobalamin (vitamin B₁₂). We hypothesized that the cblC protein would also catalyze the dealkylation of newly internalized methylcobalamin (MeCbl) and 5'-deoxyadenosylcobalamin (AdoCbl), the naturally occurring alkylcobalamins that are present in the diet. The hypothesis was tested in cultured endothelial cells using [⁵⁷Co]-AdoCbl and MeCbl analogs consisting of [⁵⁷Co]-labeled straight-chain alkylcobalamins ranging from C₂ (ethylcobalamin) to C₆ (hexylcobalamin). [⁵⁷Co]-AdoCbl was converted to [⁵⁷Co]-MeCbl by cultured bovine aortic endothelial cells, suggesting that a dealkylation process likely involving the cblC protein removed the 5'-deoxyadenosyl alkyl group. Surprisingly, all of the straight-chain alkylcobalamins served as substrates for the biosynthesis of both AdoCbl and MeCbl. Dealkylation was then assessed in normal skin fibroblasts and fibroblasts derived from three patients with mutations in the MMACHC gene. While normal skin fibroblasts readily converted [⁵⁷Co]-propylcobalamin to [⁵⁷Co]-AdoCbl and [⁵⁷Co]-MeCbl, there was little or no conversion in cblC mutant fibroblasts. These studies suggest that the CblC protein is responsible for early processing of both CNcob (decyanation) and alkylcobalamins (dealkylation) in mammalian cells.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Cobalamin (Cbl) is an essential micronutrient required by all cells in the body. Adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) serve as coenzymes for methylmalonyl-CoA mutase (EC 5.4.99.2) and methionine synthase (EC 2.1.1.13), respectively. Insufficient dietary intake of Cbl, malabsorption, defective transport, or impaired intracellular processing and coenzyme biosynthesis can lead to clinical cobalamin deficiency [1]. Accumulating evidence suggests that the intracellular processing of dietary cobalamins and cyanocobalamin (CNcobl, vitamin B₁₂) precedes the biosynthesis of AdoCbl and MeCbl [2–6]. At least one early processing step is dependent on the MMACHC gene product (hereafter referred to as the cblC protein), which is defective in patients with cobalamin disorders belonging to the cblC complementation group. The cblC complementation group, first described in a patient 4 decades ago by Mudd and colleagues [7], contains the largest number of inherited defects of cobalamin metabolism (OMIM 277400). Patients with the cblC defect usually present with combined homocystinuria and methylmalonic aciduria, suggesting impaired methionine synthase and methylmalonyl-CoA mutase activities, respectively. Lerner-Ellis et al. recently identified the gene in the cblC locus on chromosome region 1p using homozygosity mapping and haplotype analyses and named it MMACHC for “methylmalonic aciduria type C and homocystinuria” (Gene ID 25974) [5]. Cultured skin fibroblasts from patients with mutations in the MMACHC gene are unable to utilize CNcobl for the biosynthesis of AdoCbl and MeCbl [8,9]. Recently, Kim et al. discovered that the cblC protein catalyzes the reductive decyanation of CNcobl [6]. Because the alkylcobalamins MeCbl and AdoCbl are major cobalamin forms found in mammalian tissues, plasma and milk [10,11], we hypo-

* Corresponding author. Address: Department of Cell Biology, NC-10, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH 44195, USA. Fax: +1 216 444 9404. E-mail address: jacobsd@ccf.org (D.W. Jacobsen).

† Present address: School of Biotechnology, Yeungnam University, Gyeongsan 712-748, South Korea.
esized that the cbIC protein would also catalyze the dealkylation of newly internalized (diaryl) alkylcobalamins. Herein, we provide strong evidence that (1) aortic endothelial cells possess the required machinery to synthesize AdoCbl and MeCbl from both natural and xenobiotic alkylcobalamins; (2) the processing machinery responsible for dealkylation reactions displays broad substrate specificity; (3) patient fibroblasts with mutations in the MMACHC gene are unable to perform dealkylation reactions. This is consistent with a role for the involvement of the cbIC protein in dealkylation processing of newly absorbed alkylcobalamins.

Materials and methods

Synthesis and purification of ethylcobalamin (EtCbl), propylcobalamin (PrCbl), butyrycobalamin (BuCbl), pentyloxy cobalamin (PrnCbl) and hexylcobalamin (HxCbl)

Xenobiotic alkylcobalamins were synthesized by the reaction of cob(II)alamin with the corresponding alkylhalide [13,14] and purified by HPLC as described in Supplementary material (see Fig. S1, Supplementary material). [57Co]-Alkylcobalamins were synthesized as described above using [57Co]-CNcbl (MP Biomedicals, Solon, OH) as the starting material (specific activity: 379 μCi/μg). 5’-Chloro-5’-deoxyadenosine, synthesized as described by Jacobsen et al. [12], was used to synthesize [57Co]-AdoCbl.

Cell culture lines and [57Co]-cobalamin metabolic labeling

Bovine aortic endothelial cells (BAEC) were cultured in 162 cm² flasks (Corning) and grown in vitamin B12-free, folic acid-free Ham’s F12/DME (1:1) medium supplemented with 5% FBS, 2.0 mM l-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 50 nM (6S)-N⁵-methyltetrahydrofolic acid (Eprona AG). The amount of cobalamin present in the 5% FBS-supplemented culture medium (33 pm) was shown to be sufficient to support normal growth of BAEC. Normal and cbIC mutant fibroblasts were grown in Advanced DMEM (Gibco) culture medium supplemented with 10% FBS (final cobalamin concentration, 66 pm). For [57Co]-Cbl metabolic labeling experiments, cells were passaged at a ratio of 1:2. [57Co]-CNcbl was added to achieve a final concentration of 0.2 nM, and cells were grown to 100% confluence (~48 h).

cbIC patient cell lines

Dr. David Watkins, McGill University, kindly provided human cbIC mutant skin fibroblasts from patients with severe disease (WG1801, WG2176 and WG3354). The Repository for Mutant Human Cell Strains, Montreal Children’s Hospital, Montreal, Canada (http://www.cellbank.mcgill.ca/) provided patient information on the cbIC lines. Patient WG1801 was a 2-month-old male of Turkish ethnicity, son of first cousins, with two brothers who were possible carriers of the inborn error. Patient WG2176 was a 7-month-old male of Hong Kong Chinese ethnicity with a healthy older sister and an affected fetal brother who was aborted. Patient WG3354 was a female of Pakistani ethnicity with both parents and younger siblings heterozygous for the cbIC mutation.

Assessment of dealkylation in BAEC and human fibroblasts

Cells were passaged at a ratio of 1:2 in medium containing 0.125 nM (specific activity: 379 μCi/μg) of the desired [57Co]-cobalamin. After 48 h, cells were harvested, total cobalamins extracted with 80% aqueous ethanol and the intracellular cobalamin profile determined as recently described by Hannibal et al. [15]. The cell cultures were protected from light at all times to prevent photolysis of the alkylcobalamins.

Stability of [57Co]-alkylcobalamins in the culture medium

Conditioned medium (1 ml) from 48-h old cultures was extracted with a 1:1 mixture of phenol/chloroform, taken to dryness in a Speedvac, reconstituted with 0.4 ml of phosphate-buffered saline (PBS). Cobalamin standards were added to the sample, the mixture was filtered (0.22 μm filter) and analyzed by HPLC as previously described [15]. Workup of the conditioned culture medium was conducted under dim-red light.

Extraction and analysis of intracellular cobalamins

Confluent cells were harvested by trypsinization and washed three times with Dulbecco’s PBS. Extraction of cobalamins from cell pellets was performed as previously described [15]. Extracted [57Co]-cobalamins were mixed with unlabeled cobalamin standards and separated by gradient reverse-phase HPLC using an Agilent 1100 System equipped with a Zorbax SB C-18 column (4.6 × 250 mm, 5 μm particle size, Agilent) as previously described [15]. Elution was monitored with UV detection at 254 nm. In typical runs, 60 fractions were collected. The radioactivity associated with each fraction was counted using a gamma-counter (Gamma 4000 Beckman-Coulter).

Cobalamin uptake studies

Cells were seeded at an initial density of ~50% and allowed to grow for 24 h. After 24 h, half of the conditioned culture medium was replaced with fresh medium and [57Co]-CNcbl added to a final concentration of 0.2 nM (specific activity: 379 μCi/μg). Uptake was followed by counting the radioactivity in a γ-counter at 6, 12, 24, 48 and 72 h, both in spent medium and in washed cell pellets. Total cobalamin values were normalized to cellular protein concentration.

Cytotoxicity

To rule out cytotoxic effects of the xenobiotic alkylcobalamins under our culture conditions, BAEC were grown in the presence of 1 μM of each of the xenobiotic alkylcobalamins (supraphysiolog- ical concentration) as the major source of cobalamin for 2 weeks, with medium plus fresh alkylcobalamin changes every 3 days. Morphological changes were monitored by phase-contrast microscopy. To assess for cobalamin deficiency, total homocysteine and methylmalonic acid concentrations were determined in the conditioned culture medium. Cell number and cell viability were determined by hemocytometry and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16], respectively. Cellular senescence was assessed by determining β-galactosidase activity using a commercial kit (Sigma).

Biochemical analyses

Total homocysteine in conditioned culture medium was determined by the method of Jacobsen et al. using monobromobimane and HPLC with fluorescence detection [17]. Values were normalized to cellular protein concentration. The concentration of methylmalonic acid in conditioned culture medium was determined by gas chromatography and mass spectrometry (GC/MS) in the Department of Clinical Pathology, Cleveland Clinic by a method modified from Hoffmann et al. [18]. Total protein concentration was determined by the bicinchoninic acid assay (Thermo Scientific) using bovine serum albumin as a standard.
Results

Cobalamin uptake and cofactor biosynthesis by BAEC

Because little is known about B12 metabolism in cardiovascular cells and tissues, the ability of BAEC to internalize \[^{57}\text{Co}\]-CNCbl and synthesize \[^{57}\text{Co}\]-AdoCbl and \[^{57}\text{Co}\]-MeCbl was first determined. \[^{57}\text{Co}\]-CNbl was added to pre-confluent cells in culture and total cobalamin uptake was measured at 6, 12, 24, 48 and 72 h in washed cell pellets. Data for uptake of \[^{57}\text{Co}\]-CNbl by BAEC are shown in Table 1. Maximum uptake occurs at \(\sim 48\) h of growth under the culture conditions described above. For each time point, the intracellular cobalamin profile was also examined and the results for cofactor biosynthesis are summarized in Table 1. The kinetics of biosynthesis of the active cofactors, AdoCbl and MeCbl is shown in Fig. 1. Although AdoCbl appeared to be the most abundant cobalamin form at all times, the ratio AdoCbl/MeCbl varied over time. BAEC also utilized \[^{57}\text{Co}\]-hydroxocobalamin (\[^{57}\text{Co}\]-HOCbl) and \[^{57}\text{Co}\]-AdoCbl as substrates for cofactor biosynthesis (Fig. S2, Supplementary material). These results are summarized in Table 2. Thus, endothelial cells possess the machinery for the efficient conversion of the natural cofactor AdoCbl into MeCbl as well as for utilizing HOCbl and CNbl as substrates for cofactor biosynthesis (see Table 2).

Cytotoxicity of xenobiotic alkylcobalamins

It has been reported that EtCbl and PrCbl form inactive complexes with apo-methionine synthase \(\text{in vitro}\) [19]. Therefore, we examined the effects of high concentrations of each of the non-radioactive xenobiotic alkylcobalamins on cultured BAEC were examined. None of the xenobiotic alkylcobalamins appeared to be cytotoxic (Fig. S3, Supplementary material). In addition, cell viability (MTT assay), senescence (\(\beta\)-galactosidase activity), and export of homocysteine and methylmalonic into the culture medium were also examined. None of these markers were altered in the presence of xenobiotic cobalamins compared to cells supplemented with CNCbl, AdoCbl or no cobalamin at all (data not shown). The lack of cytotoxicity of EtCbl, PrCbl and the other extended alkylcobalamins suggests that they are converted to nontoxic cobalamins, which serve as substrates for the biosynthesis of MeCbl and AdoCbl.

Dealkylation of xenobiotic alkylcobalamins by BAEC

The ability of BAEC to convert a series of \[^{57}\text{Co}\]-labeled xenobiotic alkylcobalamins with increasing \(\beta\)-axial ligand alkyl chain length into the active coenzyme forms was determined. Cells were grown for \(48\) h in the presence of \(125\) pM \[^{57}\text{Co}\]-alkylcobalamin.

### Table 1

Uptake and processing of \[^{57}\text{Co}\]-CNbl by BAEC.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>[^{57}\text{Co}]-CNbl uptake(^a) (fmol/mg protein)</th>
<th>Conditioned medium</th>
<th>Intracellular</th>
<th>[^{57}\text{Co}]-Cofactor biosynthesis(b) (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AdoCbl</td>
</tr>
<tr>
<td>6</td>
<td>10.5</td>
<td>0.36</td>
<td>0.006 ± 0.002</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>12</td>
<td>8.8</td>
<td>0.44</td>
<td>0.035 ± 0.005</td>
<td>0.007 ± 0.002</td>
</tr>
<tr>
<td>24</td>
<td>8.4</td>
<td>0.54</td>
<td>0.044 ± 0.014</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>48</td>
<td>8.3</td>
<td>0.59</td>
<td>0.090 ± 0.021</td>
<td>0.012 ± 0.003</td>
</tr>
<tr>
<td>72</td>
<td>8.5</td>
<td>0.60</td>
<td>0.113 ± 0.016</td>
<td>0.008 ± 0.002</td>
</tr>
</tbody>
</table>

\(^a\) Uptake results represent three pooled samples per time point.

\(^b\) Cofactor biosynthesis is expressed as mean ± standard deviation (\(n = 3\)).

### Fig. 1

Processing \[^{57}\text{Co}\]-CNbl in BAEC. Kinetics of cobalamin biosynthesis in BAEC for 6, 12, 24, 48 and 72 h. BAEC were grown in the presence of \[^{57}\text{Co}\]-CNbl (0.2 nM final concentration; 0.1 \(\mu\)Ci/ml culture medium) as the cobalamin source. Absorbance at 254 nm for cobalamin standards is shown in the upper chromatogram. Radioactivity for \[^{57}\text{Co}\]-cobalamins at 6, 12, 24, 48 and 72 h is shown in the bottom chromatograms.
<table>
<thead>
<tr>
<th>Source</th>
<th>Intracellular cobalamin (%)</th>
<th>AdoCbl</th>
<th>MeCbl</th>
<th>HOCbl</th>
<th>CNCbl</th>
<th>Othersb</th>
</tr>
</thead>
<tbody>
<tr>
<td>58.1</td>
<td>4.5</td>
<td>20.4</td>
<td>&lt;1</td>
<td>17.0</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td>33.6</td>
<td>7.5</td>
<td>3.7</td>
<td>47.1</td>
<td>8.1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>57.1</td>
<td>8.6</td>
<td>10.2</td>
<td>&lt;1</td>
<td>23.1</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>59.1</td>
<td>5.5</td>
<td>15.4</td>
<td>&lt;1</td>
<td>20.0</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>32.7</td>
<td>8.1</td>
<td>7.7</td>
<td>&lt;1</td>
<td>51.5</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>28.8</td>
<td>4.0</td>
<td>12.4</td>
<td>&lt;1</td>
<td>54.8</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>43.2</td>
<td>7.4</td>
<td>15.1</td>
<td>&lt;1</td>
<td>34.3</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>48.5</td>
<td>8.7</td>
<td>16.2</td>
<td>&lt;1</td>
<td>26.6</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>

a BAEC were cultured in the presence of [57Co]-cobalamins for 48 h. Intracellular [57Co]-cobalamins were extracted from washed cells and separated by HPLC as described in Experimental procedures.

b “Others” includes unprocessed Cbl source and unidentified corrinoids.
Cobalamins present in cells and in the conditioned culture medium were extracted and analyzed as described under Materials and Methods. The intracellular cobalamin profile of cells grown in the presence of \(^{57}\text{Co}\)-PrCbl is shown in Fig. 2A. Cells were able to dealkylate \(^{57}\text{Co}\)-PrCbl and efficiently convert it to the two natural cofactors AdoCbl and MeCbl. Very little \(^{57}\text{Co}\)-PrCbl was recovered from the cell extracts (Fig. 2A). Examination of the 48-h conditioned culture medium (Fig. 2B) revealed a prominent \(^{57}\text{Co}\)-PrCbl peak but little or no other \(^{57}\text{Co}\)-labeled cobalamins suggesting that the \(^{57}\text{Co}\)-PrCbl substrate was stable and that there was little or no export of cellular cobalamins from BAEC into the conditioned medium. Similarly, all of the other \(^{57}\text{Co}\)-labeled alkylcobalamins used in this study were stable and did not degrade in the culture medium (data not shown). \(^{57}\text{Co}\)-AdoCbl was the major form of cobalamin found in BAEC after feeding with \(^{57}\text{Co}\)-labeled alkylcobalamins. Table 2 summarizes the results obtained for the xenobiotic alkylcobalamin series (EtCbl, PrCbl, BuCbl, PnCbl, HxCbl), as well as results from the naturally occurring cobalamin forms (AdoCbl, CNCbl and HOClb).

Genetic background, biochemical characterization and ability of the \(\text{cbl}C\) cell lines to perform decyanation of \(^{57}\text{Co}\)-CNCbl

A summary of the mutations present in the \(\text{cbl}C\) cell lines used in this study and the age of onset of the disease are presented in Table 3. The three patient \(\text{cbl}C\) cell lines present distinct mutations in the \(\text{MMACHC}\) gene, which led in all cases to a severe impairment of cobalamin metabolism. Total levels of homocysteine and methylenalonic acid were assessed in the conditioned culture medium of normal and \(\text{cbl}C\) mutant fibroblasts grown for 7 days, and are shown in Table 3. All \(\text{cbl}C\) cell lines excreted increased levels of both Hcy and MMA compared to normal fibroblasts. In addition, all \(\text{cbl}C\) cell lines were unable to decyanate \(^{57}\text{Co}\)-CNCbl and synthesize \(^{57}\text{Co}\)-MeCbl and \(^{57}\text{Co}\)-AdoCbl (Table 3). In contrast normal fibroblasts performed decyanation and subsequent cofactor biosynthesis efficiently. Patient cell line WG3354 performed decyanation to form HOClb, however, there was no detectable cofactors biosynthesis (Table 3). Overall, this is the biochemical phenotype expected for combined methylmalonic aciduria and homocystinuria, hence, the \(\text{cbl}C\) patient cell lines selected herein represent a suitable model to investigate the role of the \(\text{cbl}C\) protein in the dealkylation process.

Dealkylation of xenobiotic alkylcobalamins by normal and \(\text{cbl}C\) mutant fibroblasts

Because patients with a defective \(\text{MMACHC}\) gene are unable to utilize CNCbl as a substrate for cofactor biosynthesis [1,4,9] (Table 3), we hypothesized that \(\text{cbl}C\)-derived skin fibroblasts would be incapable of dealkylating newly internalized alkylcobalamin. To test this hypothesis, normal and \(\text{cbl}C\) mutant cell lines were incubated with \(^{57}\text{Co}\)-PrCbl as described, and after 48 h, the intracellular cobalamin profiles were examined. As shown in Fig. 3, there was a much reduced capacity for the \(\text{cbl}C\) mutant lines to convert \(^{57}\text{Co}\)-PrCbl to \(^{57}\text{Co}\)-AdoCbl and \(^{57}\text{Co}\)-MeCbl, and most of the cobalamin in the mutant cells was unprocessed \(^{57}\text{Co}\)-PrCbl. However, normal fibroblasts were very efficient at converting \(^{57}\text{Co}\)-PrCbl into \(^{57}\text{Co}\)-MeCbl, the predominate form, and, to a lesser extent, \(^{57}\text{Co}\)-AdoCbl (Fig. 3). These results are consistent with a role for the \(\text{cbl}C\) protein in removing alkyl groups from the \(\beta\)-axial ligand position of alkylcobalamins.

Table 3

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mutation 1</th>
<th>Mutation 2</th>
<th>Age of onset</th>
<th>Metabolites in conditioned culture medium</th>
<th>Decyanation of (^{57}\text{Co})-CNCbl and cofactor biosynthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hcy (nmol/mg protein)</td>
<td>MMA (nmol/mg protein)</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td>11.2 ± 11.8</td>
<td>0.76 ± 0.07</td>
</tr>
<tr>
<td>WC1801 c.217C&gt;T</td>
<td>c.217C&gt;T</td>
<td>&lt;2 months</td>
<td>52.8 ± 25.5</td>
<td>28.9 ± 3.6</td>
<td>19.5 ± 3.7</td>
</tr>
<tr>
<td>WG2176 c.234A&gt;G</td>
<td>c.690G&gt;A</td>
<td>Birth</td>
<td>92.3 ± 23.2</td>
<td>25.3 ± 4.8</td>
<td>26.4 ± 1.1</td>
</tr>
<tr>
<td>WG3354 c.435 436delAT</td>
<td>c.435 436delAT</td>
<td>&lt;2 months</td>
<td>48.1 ± 8.1</td>
<td>25.3 ± 4.8</td>
<td>26.4 ± 1.1</td>
</tr>
</tbody>
</table>

\(^a\) Values represent mean ± standard deviation \((n = 3)\). Total Hcy and MMA were determined in the conditioned culture medium of cells grown for 7 days. Differences in Hcy and MMA levels between the normal and the \(\text{cbl}C\) mutant cell lines were statistically significant, as determined by Student's \(t\)-test at the 95% level of confidence \((p < 0.05)\).

\(^b\) Values represent mean ± standard deviation \((n = 3)\).

\(^{c}\) ND, not detectable.
Discussion

The primary objective of the current work was to demonstrate that mammalian cells are capable of processing alkylcobalamins and to provide evidence that the processing is mediated by the cblC protein. Bovine aortic endothelial cells (BAEC) were used for assessing dealkylation processing and conversion to AdoCbl and MeCbl. The vascular endothelium appears to play important roles in cobalamin homeostasis. For example, endothelial cells synthesize and secrete considerable amounts of transcobalamin, the serum binding protein that delivers to the cells throughout the body [20]. However, there is little information on cobalamin processing and coenzyme biosynthesis by vascular endothelial cells. We hypothesized that the vascular endothelium is able to utilize CNCbl and alkylcobalamins as substrates for the synthesis of AdoCbl and MeCbl and that the cblC protein recently shown to catalyze the in vitro deacylation of CNCbl [6], also catalyzes the dealkylation of alkylcobalamins in BAEC.

Our results demonstrate that cultured BAEC convert CNCbl to both AdoCbl and MeCbl. The amount of AdoCbl synthesized is always greater than the amount of MeCbl synthesized (Table 1). However, there was considerable variation in the AdoCbl/MeCbl product ratio depending on the substrate. When HOCbl and CNCbl were used as substrates, the AdoCbl/MeCbl product ratio was 12.9 and 4.5, respectively. Next, we assessed the ability of BAEC to utilize AdoCbl as a substrate for the biosynthesis of MeCbl as reported for cultured human lymphocytes several years ago [3]. Since AdoCbl and MeCbl are naturally occurring alkylcobalamins in circulation [10,21], BAEC must have a system for dealkylating these endogenous alkylcobalamins that are delivered to the cell. We find that BAEC are indeed capable of converting AdoCbl to MeCbl.

We then determined whether the putative “dealkylase” activity would remove other alkyl groups from the β-axial position of cobalamins. Xenobiotic straight-chain alkylcobalamins were synthesized and purified, ranging from CH$_3$CH$_2$ - (ethylcobalamin) to CH$_3$(CH$_2$)$_{36}$- (hexylcobalamin). None of the straight-chain alkylcobalamins appeared to be cytotoxic to cultured BAEC. Surprisingly, all of the xenobiotic alkylcobalamins served as substrates for the synthesis of AdoCbl and MeCbl with AdoCbl/MeCbl product ratios ranging from 4.0 to 10.7 (Table 2). While our study demonstrates that the dealkylase system has broad substrate specificity for the ligand coordinating at the β-axial position of the cobalamin molecule, it does not address whether this activity is associated with one or more proteins.

The mystery of how decyanation of CNCbl occurs was recently solved by the in vitro studies of Kim et al. [6]. Decyanation is a process that is dependent on the activity of the CblC protein. The protein is a monomer of 29 kDa, which catalyzes the reductive decyanation of CNCbl using a flavoprotein oxidoreductase for transferring reducing equivalents from NADPH [6]. Human fibroblasts that carry mutations in the MMACHC gene exhibit impaired cobalamin processing of CNCbl and little or no AdoCbl and MeCbl biosynthesis [4,22]. Three cblC mutant fibroblasts isolated from severely ill and genetically unrelated patients were used in the present study to assess dealkylation in vivo. The biochemical profile of the cblC mutant cell lines WG1801, WG2176 and WG3354 resembled that reported for other cblC cell lines, i.e., substantial export of Hcy and MMA into culture medium (indicative of functional cobalamin deficiency) and poor or negligible utilization of CNCbl as a substrate for coenzyme biosynthesis. The inability of the cblC mutant fibroblasts to utilize [15Co]-PrCbl as a substrate for AdoCbl and MeCbl biosynthesis is consistent with the hypothesis that the cblC protein catalyzes the dealkylation. In contrast, normal fibroblasts were able to use PrCbl efficiently to generate both AdoCbl and MeCbl. Based on the observations that (1) human recombinant MMACHC catalyzes decyanation of CNCbl [6]; (2) cblC mutant fibroblasts are unable to utilize CNCbl as a substrate for coenzyme biosynthesis [4,23]; (3) cblC mutant fibroblasts are unable to perform dealkylation of [15Co]-PrCbl (this study), we propose that the cblC protein is responsible for catalyzing the removal of alkyl groups from the β-axial position of alkylcobalamins.

The phenotypic expression of combined hyperhomocysteinemia and methylmalonicacidemia is associated with patients from the cblB, cblC and cblD complementation groups. Of these, cblD is unlikely to be a dealkylase since its impairment leads to accumulation of newly internalized B$_12$ in lysosomes [24–26]. Recent work now shows that the cblD gene product is a B$_12$-lysosomal membrane transporter [27]. The cblD locus is complex since it can lead to either isolated or combined defects in methionine synthase and methylmalonyl-CoA mutase [4,28] and, for this reason, is also unlikely to encode a dealkylase that is shared by both AdoCbl and MeCbl synthesis pathways. Hence, the cblD locus appears to be the most likely candidate for encoding an alkylcobalamin dealkylase function.

The current work shows that newly internalized alkylcobalamins undergo dealkylation processing, a likely prerequisite for generating the biologically active cobalamin forms AdoCbl and MeCbl, and that the dealkylase activity requires the cblC protein. In light of our results and previous findings, alternative mechanisms for cobalamin processing in vivo should be considered. Our current thoughts on the possible mechanisms by which the cblC protein could process newly internalized natural and xenobiotic alkylcobalamins are summarized in Fig. 4. Binding of the natural alkylcobalamins MeCbl and AdoCbl to the MMACHC chaperone has been shown to induce the “base-off” conformation in which the β-axial dimethylbenzimidazole ligand is not coordinated to the cobalt. This could be important in enhancing the reactivity of the β-axial ligand [6]. A variety of mechanistic alternatives could be considered for the removal of an alkyl group from the β-axial position of cobalamins (Fig. 4). First, homolysis of the cobalt–carbon bond would generate cob(I)alamin and an alkyl radical (Reaction 1, Fig. 4). Second, nucleophilic displacement of the alkyl group would result in the formation of cob(II)alamin and the transfer of the alkyl carbocation to the acceptor (Reaction 2, Fig. 4) [29,30]. Third, reductive dealkylation could occur resulting in the
formation of either cob(II)alamin or cob(I)alamin and the departure of the alkyl group as a carbanion or a radical, respectively.

Acknowledgment

This research was funded by the National Heart, Lung and Blood Institute of the National Institutes of Health (HL71907 to D.W.J.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymgme.2009.04.005.

References


