

Identification of multidrug resistance protein 1 (MRP1/ABCC1) as a molecular gate for cellular export of cobalamin

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Cobalamin (Cbl, vitamin B₁₂) deficiency in humans is a cause of hematologic and neurologic disorders. We show here that the cellular export of Cbl, in contrast to the carrier- and receptor-dependent cellular import of Cbl, occurs by transmembrane transport of “free” Cbl. Screening of candidate transporters by cellular gene silencing showed a role in cellular Cbl efflux of the ATP-binding cassette (ABC)–drug transporter, ABCC1, alias multidrug

resistance protein 1 (MRP1), which is present in the basolateral membrane of intestinal epithelium and in other cells. The ability of MRP1 to mediate ATP-dependent Cbl transport was confirmed by vesicular transport experiments, and a physiologic role of MRP1 in mammalian Cbl homeostasis is indicated by the phenotype of knockout mice with targeted disruption of MRP1. These animals have a reduced concentration of Cbl in plasma

and in the storage organs liver and kidney. In contrast, Cbl accumulates in the terminal part of the intestine of these mice, suggesting a functional malabsorption because of a lower epithelial basolateral Cbl efflux. The identification of this Cbl export mechanism now allows the delineation of a coherent pathway for Cbl trafficking from food to the body cells. (Blood. 2010;115:1632-1639)

Introduction

Cobalamin (Cbl, vitamin B₁₂) is a multifunctional organic tetrapyrrole structure synthesized by bacteria and archaea. Mammals take up Cbl from their diet because they cannot synthesize Cbl and need this vitamin as an essential coenzyme for methylmalonyl coenzyme A mutase and methionine synthase. Deficiency of Cbl is the most common vitamin deficiency disease in industrialized countries and the cause of pernicious anemia, a serious disorder characterized by megaloblastic anemia and neurologic symptoms (for review see Reynolds¹ and Depeint et al²). Deficiency of Cbl also causes a functional folate deficiency because of the role of methionine synthase in folate methylation (for review see Shane and Stokstad³).

Several proteins are involved in the uptake and transport of Cbl in mammals. In the gastrointestinal tract Cbl is bound to intrinsic factor (IF) secreted from the gastric epithelium, and the Cbl-IF complex is taken up by endocytosis after binding to the cubilin/amnionless receptor complex (cubam) present on the apical surface of the ileal epithelium.^{4,5} On internalization, IF is degraded in lysosomes and Cbl is released to the cytosol.⁶ Recent evidence from genetic analysis of a group of Cbl-deficiency patients with lysosomal accumulation of Cbl suggests that the lipocalin receptor-like protein LMBD1 is involved in Cbl transport across the lysosomal membrane.⁷

In plasma and interstitial fluids Cbl is bound to transcobalamin (TC) a protein homologous to IF. Cells internalize the TC-Cbl complex by an endocytic receptor, recently identified as the CD320 membrane protein.⁸ TC-Cbl filters through the kidney glomeruli and is efficiently taken up from the ultrafiltrate via endocytosis by binding to another receptor, megalin.⁹ Like CD320 and cubam, megalin causes the transfer of the

TC-Cbl complex to the lysosomes where the protein moiety is degraded. Cbl is exchanged between cells and organs in the body, but it is not known how intracellular Cbl is exported from cells, including enterocytes. Because enterocytes have a high rate of synthesis of TC, it has been a common view^{10,11} that Cbl is secreted from the enterocytes in complex with TC. An alternative hypothesis is that free Cbl is transported from the cytosol across the basolateral cell surface into plasma, where it subsequently forms a complex with circulating TC. Whatever the pathway for cellular Cbl excretion, it has to involve transport across the plasma membrane or a vesicular membrane.

Prokaryotes are known to import nonprotein bound (“free”) Cbl in an ATP-dependent manner by a protein belonging to the ATP-binding cassette (ABC) family (BtuCD),¹²⁻¹⁴ but no eukaryotic transporters capable of transporting free Cbl across the plasma membrane have been identified so far. Here, we report that free Cbl is effluxed by cultured cells. With a small interfering RNA (siRNA)–based screening assay we identified ABCC1 (also known as multidrug resistance protein 1; MRP1) as a transport protein, capable of effluxing free Cbl. We demonstrate that MRP1 is able to transport Cbl in vesicular transport assays and that Cbl homeostasis is altered in mice that lack MRP1.

Methods

Materials and cell lines

DB M-270 amine magnetic beads were from Dynal Biotech. Colabeled cyano-Cbl ([⁵⁷Co]-CN-Cbl) was from MP Biomedicals, and radiolabeled hydroxycobalamin ([⁵⁷Co]-OH-Cbl) was prepared from [⁵⁷Co]-CN-Cbl

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according to the method of Mahoney and Rosenberg.¹⁵ The cell lines used in the present study were differentiated and Epstein-Barr virus-immortalized Brown Norway rat yolk sac epithelial carcinoma cells (designated BN16¹⁶), human cervical cancer cell line (HeLa), Chinese hamster ovary (CHO) cells, and primary cultures of human dermal fibroblast cells and fibroblasts from mice. CHO cells were stably transfected with human TC cDNA¹⁷ (kind gift from Dr Sergey Fedosov, University of Aarhus) inserted in the pSecTag2 B vector (Invitrogen) containing the hygromycin resistance gene. HEK293 cells expressing MRP1 were generated by transfecting HEK293 cells with pCMVneoMRP1 by calcium phosphate precipitation.¹⁸ After 48 hours, cells were split, and G418 (800 µg/mL) was used to select for clones that express MRP1. G418-resistant clones were tested for MRP1 expression by immunoblot analysis with the use of the MRP1 antibody.¹⁹ Plasma membrane localization of MRP1 was confirmed by immunofluorescence microscopy with the use of the same antibody (not shown). HEK293 parental and HEK293-MRP1 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 100 U of penicillin/streptomycin/mL.

Cellular Cbl efflux assay

Subconfluent cells were grown in tissue culture flask until approximately 80% confluence. Cells were incubated with 40 pg/mL [⁵⁷Co]-CN-Cbl in complex with recombinant IF or TC¹⁷ for 24 hours. The cells were subsequently washed 3 times with sterile phosphate-buffered saline, and the medium was changed to DMEM (Invitrogen) containing 1% glutamine, 1% penicillin, and 1% streptomycin, with or without 1 µM CN-Cbl (Sigma-Aldrich). After 24 hours the medium was subjected to gel filtration (on Sephadex G25M column; GE Healthcare) or analyzed by size exclusion chromatography on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) with the use of a Dionex equipment (Dionex). Cells were collected after trypsination. TC and haptocorrin bound ⁵⁷Co-Cbl was removed from the cell culture medium with the use of magnetic beads (DynaL Biotech) coated with monoclonal antibodies against TC and haptocorrin as previously described.²⁰

RNA interference studies and RT-PCR analysis

All siRNA treatments were performed with Dharmacon ON-TARGETplus SMART pool siRNA probes (Dharmacon), using ON-TARGETplus siCONTROL Nontargeting Pool as a control (Dharmacon; D-001810-10-20). ON-TARGETplus SMART pool siRNA for ABCB6 (Dharmacon; L-007304-00), MRP1 (Dharmacon; L-007308-00), MRP3 (Dharmacon; L-007312-00), MRP5 (Dharmacon; L-007314-00), and ABCG2 (Dharmacon; L-009924-00) were transfected at a final concentration of 100nM into exponentially growing HeLa cells with DharmaFECT reagent 1 (Dharmacon; T-2001-03), according to the instructions of the manufacturer. Ninety-six hours after siRNA transfection, cells were subjected to the above-described pulse/chase experiments, and at the end of the experiment cells were harvested for the analyses of mRNA expressions by reverse transcription-polymerase chain reaction (RT-PCR) with the use of an avian-enhanced RT-PCR kit (Sigma-Aldrich). Oligonucleotides for ABC-A2 (GenBank accession no. AAK14335) RT-PCR analysis were 5'-TTCTCTGGGCTGCTGCTGAGCT-3' and 5'-AGGCCGCCCCAGAGCTGTACGAA-3'. For ABC-G2 (GenBank accession no. AAO14617) they were 5'-GGAGATGTTCTGATAAATGGAGC-3' and 5'-CTGCCACTTTATCCAGACCTAAC-3', for ABC-B9 (GenBank accession no. AAF89993) they were 5'-AAGCATGGATCAGTTCAGCACGG-3' and 5'-TGTTCAGTCTGGCAAATATGAGG-3', for ABC-B6 (GenBank accession no. AAH43423) they were 5'-TTCTGTGCATGAGTCTTTACCT-3' and 5'-TAGCGTCTCTCTGTGTGTTTCATA-3', for MRP3 (GenBank accession no. AAH50370) they were 5'-CGGACCTTTTTACTTCCAT-3' and 5'-TAAAAGGATCTTGGAGCGGAATG-3', for MRP1 (GenBank accession no. ABN79590) they were 5'-CTTCTTCTCAAGCCATCCACG-3' and 5'-CTGGGGCCCTCGTGTCATTAC-3', for PLP2 (GenBank accession no. AAI09067) they were 5'-CCTTGCTGCTATTTTCTTTG-3' and 5'-CTCTCTCAACAAGGACAACA-3', for LAPTM4B (GenBank accession no. AAO64358) they were 5'-CTGCTGTGCTGCGCATGTC-3' and

5'-GCATCATCCATGAACTCAA-3', and for CD63 (GenBank accession no. CAG46893) they were 5'-ATGGCGGTGGAAGGAGGAAT-3' and 5'-GCCCCCTGGATTATGGTCTG-3'. For β-actin (GenBank accession no. AAA51578) message analysis RT-PCR primer sequences were 5'-CGTGATGGTGGGCATGGGTGTCAG-3' and 5'-TAATGTCACGCACGATTCC-3'. All RT-PCR reactions were performed following the manufacturer's instructions. Cycles were performed at 50°C for 50 minutes, 94°C for 5 minutes, 94°C for 45 seconds, 55°C for 90 seconds, 72°C for 90 seconds (35 cycles), and 72°C for 10 minutes. PCR products were separated on a 2% agarose gel containing ethidium bromide. A 100-base pair DNA ladder standard was used as a size marker.

Western blotting

Proteins from cell lysates were separated by 4% to 16% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with the use of Sequi-blot polyvinylidene difluoride membranes (Bio-Rad). MRP1 was detected with monoclonal antibody MRPr1 (5 µg/mL),¹⁹ and β-tubulin with an anti-β-tubulin antibody (1:50 000; Sigma-Aldrich), followed by a rabbit anti-rat horseradish peroxidase-conjugated antibody (1:1000; Dako-Cytomation) and visualization by enhanced chemiluminescence.

Preparation of membrane vesicles and vesicular transport assays

Vesicles were prepared from control and human MRP1-overproducing HEK293 cells. Cells were harvested by centrifugation at 500g for 5 minutes. The pellet was resuspended in ice-cold hypotonic buffer (0.5mM sodium phosphate, 0.1mM EDTA, pH 7.4) supplemented with a protease inhibitor cocktail (Roche) and incubated at 4°C for 90 minutes. The cell suspension was homogenized with the use of a tight-fitting Dounce homogenizer. After centrifugation at 500g at 4°C for 10 minutes, the supernatant was collected and centrifuged at 4°C at 100 000g for 40 minutes. The pellet was suspended in 10mM Tris-HCl pH 7.4 and passed through a 27-gauge needle 25 times. The vesicles were dispensed in aliquots, snap-frozen in liquid nitrogen, and stored at -80°C until use.

ATP-dependent transport of [⁵⁷Co]-Cbl metabolites and [³H]-estradiol-17-β-glucuronide (E₂17βG) into inside-out membrane vesicles was measured with the rapid filtration technique as described previously.²¹ In short, vesicles were filtrated with the use of a MultiScreen_{HTS} vacuum manifold in combination with Multiscreen_{HTS} FB 96-well filter plates (Millipore). Membranes were washed 4 times with 200 µL of ice-cold phosphate-buffered saline, and the radioactivity retained on the membranes was counted by liquid scintillation counting ([³H]-E₂17βG) or γ-counting ([⁵⁷Co]-Cbl metabolites). Glutathionyl-Cbl (GS-Cbl) was synthesized as previously described.²²

Animals

Mrp1^(-/-) mice have been described previously²³ and were on a 99% Friend virus B-type background. Mice received food and water ad libitum and were housed in constant temperature rooms with a 12-hour light/12-hour dark cycle. Mouse handling and experimental procedures were conducted in accordance with institutional guidelines for animal care and use and approved by the Institutional Review Board for Animal Care and Use Committee, the Netherlands Cancer Institute.

Determination of the concentration of Cbl, methylmalonic acid, and homocysteine in mouse plasma, liver, kidney, and intestine

Concentrations of Cbl in plasma and homogenized tissues of 10 to 14 weeks old, age- and sex-matched mice were determined with the use of a commercially available competitive protein binding assay with IF as the binding protein (ACS; Centaur Automated Chemiluminescence System; Bayer A/S, Diamond Diagnostics). Concentrations of plasma folate were measured on the same platform. Levels of plasma methylmalonic acid and homocysteine were measured with the use of gas chromatography-mass spectrometry after derivatization with methylchloroformate as previously described.²⁴

Results

Cellular efflux of Cbl

To measure cellular efflux of Cbl we loaded cells with ^{57}Co -labeled Cbl and followed the efflux after washing. Rat yolk sac epithelial cells, which contain the IF-Cbl receptor (cubam), were loaded with the Cbl-IF complex; fibroblasts/HeLa cells, which contain the TC receptor, were loaded with the TC-Cbl complex. After wash and chase of the cells for 24 hours, most of the radioactivity was present in the medium. Gel filtration analysis (Figure 1A) of the medium showed that the vitamin migrated as a high-molecular-weight complex and subsequent high-resolution high-performance liquid chromatography gel filtration of this material showed that the elution profile of the [^{57}Co]-label in the medium corresponded to that of Cbl bound to TC (Figure 1A). This was confirmed using established immunoprecipitation assays for TC and haptocorrin (another Cbl binder in humans).¹⁷ This showed that 85% to 90% of the ^{57}Co -Cbl activity was bound to anti-TC antibody beads (Figure 1B). Including saturating amounts of unlabeled Cbl (1 μM) in the culture medium in the chase phase resulted in the appearance of [^{57}Co]-labeled Cbl in its free form (Figure 1A left, open circle). Similar results were obtained with the use of TC-transfected CHO cells (which have a high endogenous production of TC; supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The free Cbl detected in the medium after a chase with excess Cbl was not derived from the dissociation of Cbl from the TC-Cbl complexes, because we found that less than 10% of the labeled Cbl in the TC-Cbl complex dissociated in cell medium within 24 hours at 37°C (data not shown) in agreement with previous observations.²⁵ In summary, this set of experiments indicates that cells efflux Cbl in a free form, but after secretion, Cbl binds to TC released by the cells.

Screening for candidate proteins mediating Cbl efflux

To identify the transport protein involved in the efflux of free Cbl, we focused on ABC transporters because of their structural similarity to the known prokaryotic Cbl ABC transporter BtuCD.²⁶ The ABC transporters selected for further investigation matched 2 of the following 3 criteria: (1) the ability to transport porphyrin structures (similarity to the corrin structure of Cbl), (2) a basolateral localization in polarized epithelial cells (such as enterocytes and yolk sac cells), and (3) a wide tissue distribution. This bioinformatic approach led to the selection of 5 ABC proteins: ABCB6, ABCG2, MRP1 (ABCC1), MRP3 (ABCC3), and MRP5 (ABCC5) of which ABCB6, ABCG2, and MRP3 have all been reported to transport porphyrins,²⁷ and MRP1, MRP3, and MRP5 are expressed in basolateral surfaces of various polarized cells.^{28,29} Furthermore, they all have a relatively wide tissue distribution, although MRP5 is present at low levels (for review see Borst et al,²⁹ Deeley et al,³⁰ Borst and Elferink,³¹ and Kruh et al³²).

Human HeLa cells, which contain detectable mRNA levels (Figure 2A) for all of the candidate ABC proteins (but as expected a very low signal for MRP5), were used for siRNA-mediated ABC transporter mRNA knockdown experiments. RNA transcript levels were measured by RT-PCR band intensity. ABCB6, ABCG2, MRP1, and MRP3 RNAs could be reduced by greater than 80% by transcript-specific siRNAs transfections and for MRP5 to a level below detection (Figure 2). Knockdown of the MRP1 transcript resulted in greater than 50% reduction in the amount of ^{57}Co -Cbl effluxed by the cells and a corresponding increase in the amount of

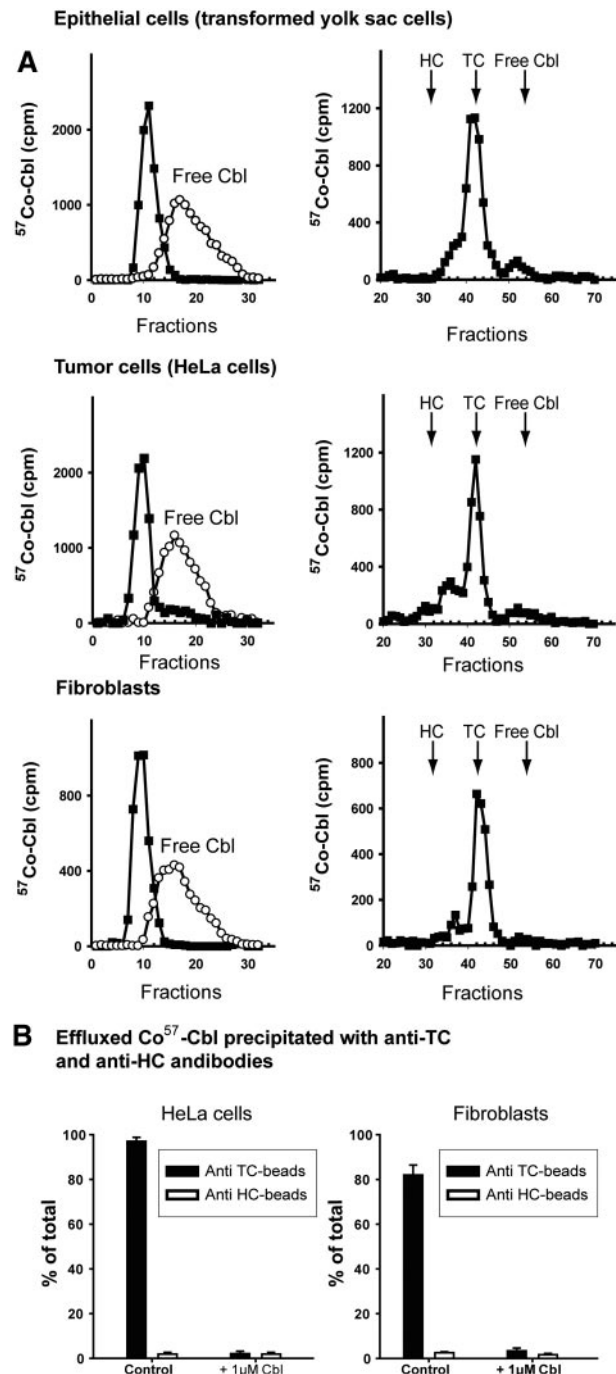
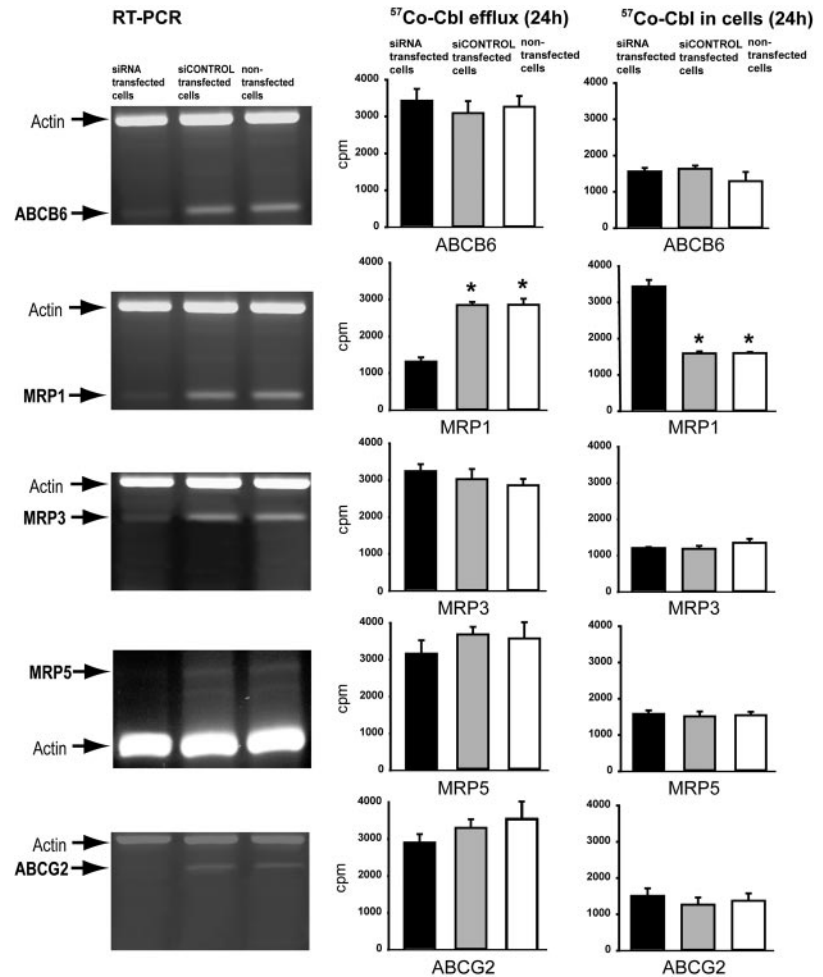


Figure 1. Analysis of ^{57}Co -Cbl released from cells. (A left) Gel filtration chromatography of ^{57}Co -Cbl in 24 hours of chase medium from cells loaded with ^{57}Co -Cbl. The chase of the cells was performed in the absence (■) or presence (○) of 1 μM Cbl in the chase medium. (A right) Analysis of the high-molecular-weight complex-form of ^{57}Co -Cbl in a high-resolution SMART high-performance liquid chromatography gel filtration system (Superdex 200 column). ↓ indicates known position of Cbl in complex with haptocorrin (HC), transcobalamin (TC), as well as free Cbl. (B) Precipitation of ^{57}Co -Cbl in 24 hours of efflux medium of the human cells (HeLa cells and fibroblasts) with magnetic beads coated with monoclonal antibodies against human TC or human HC. Precipitation was carried out in chase medium with or without 1 μM Cbl added at the initiation of the chase. The values are the means of triplicates \pm 1 SD.

Cbl present intracellularly (Figure 2 right). Western blotting confirmed that the knockdown had reduced the level of MRP1 protein greater than 80% (supplemental Figure 2). Similar results were obtained after knockdown of MRP1 in human fibroblasts and

Figure 2. siRNA silencing of mRNA encoding 5 candidate proteins for efflux of Cbl. (A) The left lanes show RT-PCR analysis of ABCB6, MRP1, MRP3, MRP5, and ABCG2 RNA in HeLa cells incubated for 96 hours with specific siRNA probes targeting the mRNA encoding the indicated membrane transporters. The middle lanes show the same analysis of cells incubated with siCONTROL and the right lanes show the analysis of nontransfected cells. (B) ^{57}Co -Cbl 24-hour efflux (left) and cellular accumulation (right) of HeLa cells transfected with the siRNA probes for ABCB6, MRP1, MRP3, MRP5, and ABCG2 mRNA (■). □ and ▨ show the same data for cells transfected with siCONTROL and nontransfected cells, respectively. All data are shown as mean of triplicates \pm 1 SD. Asterisks indicate the *P* values less than .001 (*t* test) for the difference between transfected and nontransfected cells.



the monkey COS-1 epithelial cell line (data not shown). Knock-down of ABCB6, ABCG2, MRP3, and MRP5 did not significantly affect the Cbl efflux (Figure 2).

ATP-dependent transport of Cbl by MRP1 in vitro

Vesicular transport assays showed that MRP1 transports OH-Cbl in a time- and ATP-dependent manner (Figure 3A). MRP1-mediated transport of OH-Cbl was linear for at least 20 minutes (Figure 3A) and followed Michaelis-Menten kinetics. Nonlinear regression analysis yielded K_m and V_{max} values of $23\mu\text{M}$ and $76\text{ pmol/mg per minute}$, respectively (Figure 3B). Cbl is found in several forms intracellularly, but we were unable to synthesize all these Cbl forms in a radiolabeled form. However, in vesicular transport experiments, competition with a known substrate is often used to screen for new substrates competing for the same binding/transport site on the transporter. We, therefore, tested whether OH-Cbl, CN-Cbl, and GS-Cbl competed with the established MRP1 substrate $\text{E}_2\text{17}\beta\text{G}$ for transport (Figure 3C). Each of the known intracellular Cbl forms was found to inhibit $\text{E}_2\text{17}\beta\text{G}$ transport. GS-Cbl was the strongest inhibitor, and the results presented in Figure 3D and E clearly show that the inhibition is competitive, with a K_i of approximately $6.2\mu\text{M}$ ($\pm 1.6\mu\text{M}$). This indicates that GS-Cbl has a relatively high affinity for MRP1.

Cbl homeostasis and cellular Cbl efflux in MRP1-deficient mice

To test whether MRP1 has a physiologic role in the homeostasis/tissue disposition of Cbl, we determined Cbl levels in plasma, liver, kidney, and intestine of *Mrp1*^{-/-} and wild-type (WT) mice. The

absence of MRP1 in the *Mrp1*^{-/-} mice resulted in a reduction of the Cbl levels in plasma ($\sim 50\%$), liver ($\sim 20\%$), and kidney ($\sim 75\%$; Figure 4). In contrast, Cbl levels in the ileum and colon tissue were higher in *Mrp1*^{-/-} than in WT mice (Figure 4). The reduced plasma levels of Cbl in *Mrp1*^{-/-} mice did not lead to a significant increase in the plasma levels of methylmalonate and homocysteine (data not shown), the clinical indicators of human Cbl deficiency. The latter fits previous reports describing that *Mrp1*^{-/-} mice are healthy and do not show an overt deficiency phenotype.^{23,33,34} We also determined Cbl levels in plasma, liver, and kidney in *Mrp3*^{-/-} and *Mrp1*^{-/-}/*Mrp3*^{-/-} double knockout mice but did not find any difference in Cbl load compared with WT and *Mrp1*^{-/-} mice, respectively (data not shown).

We also used the transgenic mice to verify at the cellular level that MRP1 contributes to Cbl homeostasis by comparing the efflux of Cbl from fibroblasts derived from a *Mrp1*^{-/-} and a WT mouse. The results in Figure 5A show that Cbl efflux is diminished and Cbl accumulation is increased in the *Mrp1*^{-/-} cells in agreement with the data in Figure 2. Transfection of a MRP1 cDNA construct into the *Mrp1*^{-/-} cells rescued Cbl efflux to levels comparable to those of WT mouse fibroblasts (Figure 5B).

Discussion

In the present study, we show that Cbl is released from cells in a nonprotein bound "free" form and that MRP1 is a physiologically

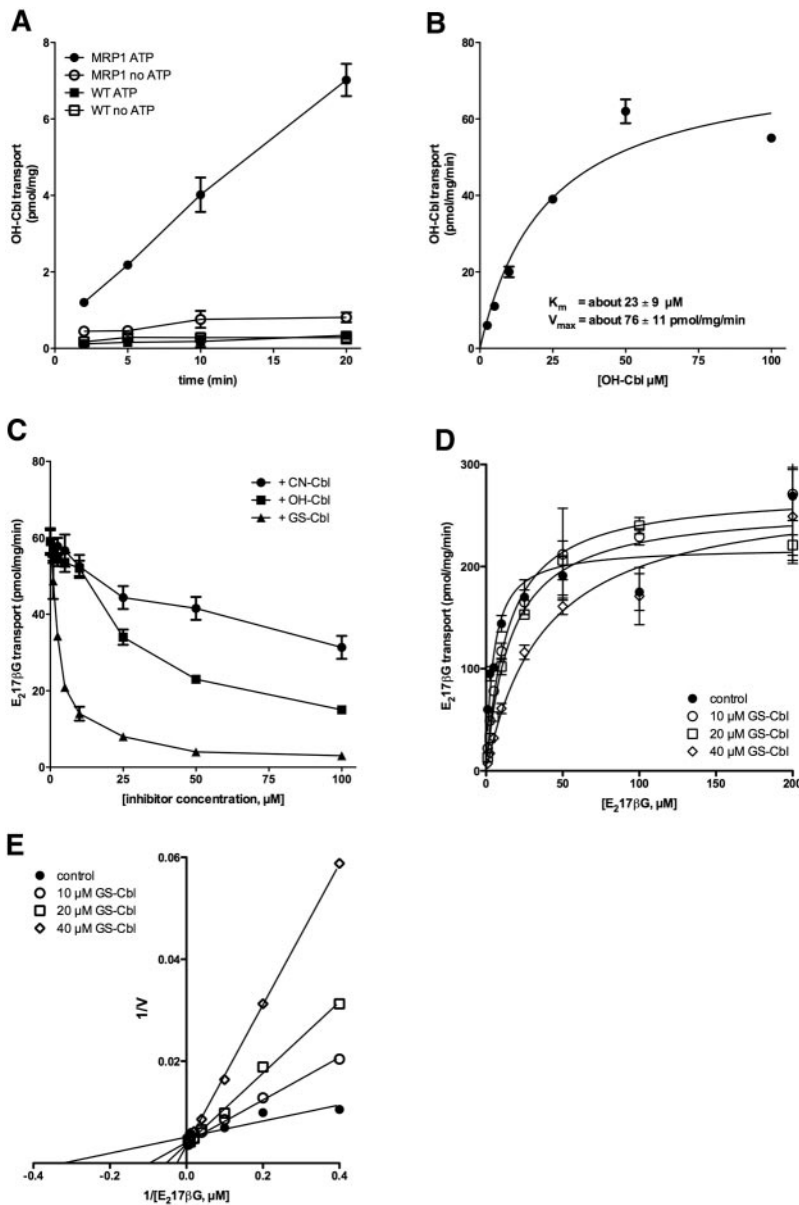


Figure 3. Transport of Cbl metabolites by MRP1 in vesicular transport experiments. (A) Time course experiments of control and MRP1-containing vesicles incubated with 100nM [^{57}Co]-OH-Cbl. (B) Concentration-dependent transport of [^{57}Co]-OH-Cbl in MRP1-containing vesicles. (C) Inhibition of MRP1-mediated transport of 1 μM [^3H]-E $_2$ 17 β G by various concentrations of unlabeled GS-Cbl, OH-Cbl, and CN-Cbl, as indicated. (D) Concentration-dependent transport of [^3H]-E $_2$ 17 β G in MRP1-containing membrane vesicles in the presence or absence of the indicated concentrations of GS-Cbl. (E) Lineweaver-Burk transformations of the data presented in panel D. Each data point represents the means \pm 1 SD of an experiment performed in triplicate. MRP1-dependent transport was calculated by subtracting ATP-dependent transport in control vesicles from ATP-dependent transport in MRP1-containing vesicles.

relevant Cbl efflux transporter for ATP-dependent cellular Cbl efflux. Coherent transcellular Cbl transport pathways can now for the first time be depicted at the molecular level (Figure 6).

Mice deficient for MRP1 have substantially less Cbl in plasma, liver, and kidney but an increased content in the intestine. This may suggest that a reduced basolateral Cbl export from the intestinal epithelium causes a reduced loading of other tissues of these mice. A precedent for the deficiency of a nutrient caused by an impaired cellular export system is the recently identified iron deficiency caused by the inactivation of the iron exporter, ferroportin, present in the basolateral surface of the intestinal epithelium and in macrophages.³⁷ The most profound effect on Cbl load by MRP1 deficiency was seen in the kidneys, which had a Cbl content of approximately 25% of that found in the WT mice. This in accordance with the finding that the kidney, which takes up Cbl from TC-Cbl complexes filtered in the renal glomeruli,⁹ is a short-time storage organ of nonenzyme-bound Cbl. A similar reduction in the kidney Cbl levels has been seen in rodents on a diet low in Cbl.³⁸ The liver Cbl content, which is largely enzyme bound³⁹ and less sensitive to the overall Cbl

body load, was accordingly only reduced by approximately 25% in the *Mrp1*^(-/-) mice.

The reduced Cbl content in plasma, liver, and kidney in *Mrp1*^(-/-) mice was accompanied by higher levels in the ileum and colon. In ileum and colon, MRP1 is present at the basolateral surface of the epithelial cells,⁴⁰ and we propose that the absence of MRP1 reduces the fraction of the Cbl taken up from the intestinal lumen that is subsequently released over the basolateral membrane toward the blood circulation. However, we found higher levels of Cbl in the colon than in the ileum, the primary site for the uptake of dietary Cbl complexed to IF in humans.⁵ How Cbl ends up in the mouse colon and whether it derives from Cbl produced in the bacterial flora of colon are unknown. The functional importance of MRP1 in the colon has been shown by the increased accumulation of etoposide in the colon of mice lacking MRP1.⁴¹

Although we attribute the lower levels of Cbl in plasma, liver, and kidney of *Mrp1*^(-/-) mice to the absence of MRP1 in the gut, we have no direct evidence yet for this hypothesis, and caution should be taken when translating data of Cbl transport from mice to man. We tested the uptake of [^{57}Co]-labeled HO-Cbl after oral

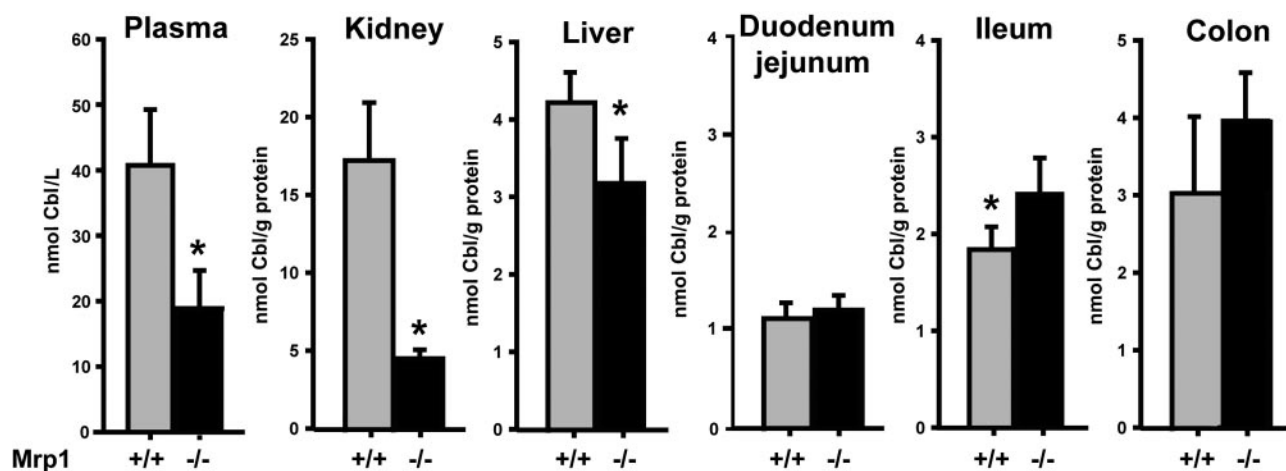


Figure 4. Cbl homeostasis in male and female MRP1-deficient mice and controls. The content of Cbl in blood plasma, kidney, liver, ileum, and colon was determined in control and *Mrp1*^{-/-} mice. The content in plasma is shown as Cbl concentration (in nM) and in the other tissues as nanomolar Cbl per gram of tissue protein. Each measurement is the mean \pm 1 SD of groups of 8 mice (4 males and 4 females). Asterisk indicates the *P* values less than .05 (*t* test) for the difference between means in the tissues.

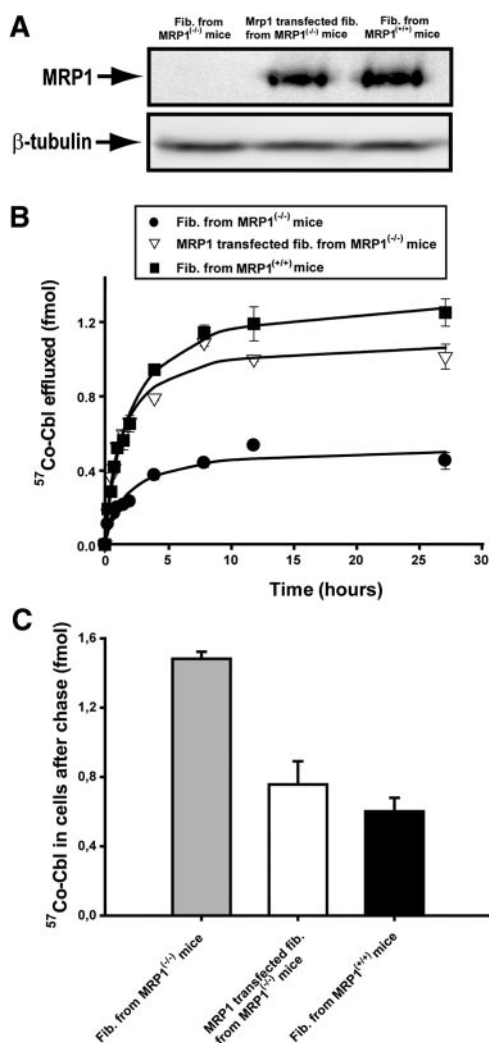


Figure 5. Time course of cellular ⁵⁷Co-Cbl efflux from MRP1-deficient and control fibroblasts. (A) MRP1 levels in skin fibroblasts from *Mrp1*^{-/-} mice, MRP1 cDNA-transfected *Mrp1*^{-/-} mice and in WT skin fibroblast. The Western blots were probed with a MRP1-specific monoclonal antibody (top), before the blot was stripped and reprobed with an antibody against β -tubulin (bottom). The cellular protein loads are the same for each lane. (B) Time course of efflux of ⁵⁷Co-Cbl in the 3 cell types. The result is normalized to cellular protein content. (C) Cellular content of ⁵⁷Co-Cbl after efflux. The values are mean \pm 1 SD of triplicate determinations.

administration, but the fraction of uptake in mice was below 10% in both WT and *Mrp1*^{-/-} mice (data not shown), and the results of this experiment were therefore not conclusive. A low specific activity of the ⁵⁷Co-HO-Cbl preparation resulting in high Cbl concentrations that saturate the Cbl receptor in the intestine may explain the low relative amount of [⁵⁷Co]-labeled HO-Cbl taken up.

Our observation that cells are able to secrete free Cbl seems in contrast with the literature,^{10,11,42} because the predominant view has been that Cbl is released from TC-producing cells in complex with TC. It should be noted, however, that efflux of free Cbl has always been an implicit assumption in the classic Shilling test, used in the clinic for evaluation of intestinal Cbl uptake. In this test, a small oral dose of [⁵⁷Co]-Cbl is administered followed by the injection of a large dose of unlabeled Cbl to prevent association of [⁵⁷Co]-Cbl with Cbl-binding proteins in the plasma to allow the determination of the amount of labeled free Cbl excreted in the urine. The fact that orally administered [⁵⁷Co]-Cbl ends up in the urine in persons with normal cubam-mediated intestinal uptake of [⁵⁷Co]-Cbl-IF and receptor-mediated uptake of Cbl-TC in the renal proximal tubules^{9,43} is therefore a strong indication that free [⁵⁷Co]-Cbl is released from intestinal cells and filtered in the glomeruli in a nonprotein bound form.

Intracellular Cbl is present in various forms, and it has been suggested that the high intracellular concentration of glutathione may cause a large part of cytosolic Cbl to be conjugated to glutathionyl groups (GS-Cbl).^{44,45} Direct experimental evidence for this plausible hypothesis remains to be provided. Although we were able to demonstrate transport of radiolabeled OH-Cbl and CN-Cbl by MRP1 in direct transport experiments, this was not possible for GS-Cbl because we were not able to make a stably radiolabeled GS-Cbl preparation (GS-Cbl transforms to OH-Cbl in water). However, unlabeled GS-Cbl most potently inhibited transport of the prototype MRP1 substrate E₂17 β G, indicating that GS-Cbl is transported by MRP1 with high affinity. Because MRP1 is a high-affinity transporter of many GSH conjugates (reviewed in Deeley and Cole⁴⁶), it is possible that GS-Cbl is a major form of Cbl transported by MRP1. It is clear from the efflux curve of Figure 5, however, that a fraction of radiolabeled Cbl loaded in the cells was not accessible for efflux and remained in the cells. The nature of this fraction is not known yet.

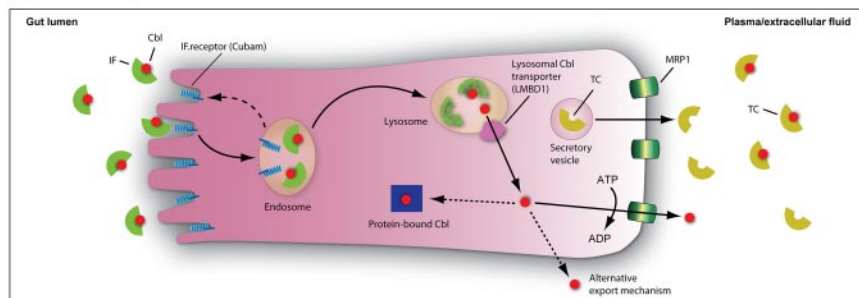
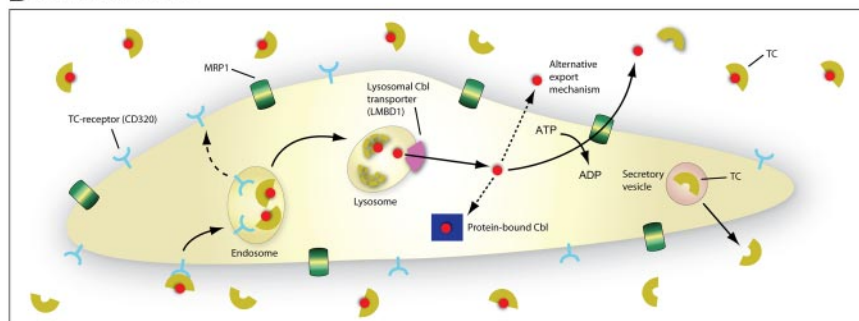
A Polarized epithelial cell**B Non-polarized cell**

Figure 6. A schematic model for the tentative cellular pathways of Cbl. (A) Polarized ileal cell. After binding of Cbl to gastric IF in the gut lumen, the IF-Cbl complex binds to the IF-Cbl receptor, cubam, located in the ileal apical brush border. Once bound, IF-Cbl is internalized and processed via the classic endosome-lysosomal pathway. In the endosome IF-Cbl is released from cubam, which is recycled back to the cell surface in receptorsomes (dashed line). In the lysosome, the lysosomal proteases degrade IF, releasing the vitamin for transport by the lysosomal transmembrane protein LMBD1. From the cytosol Cbl is exported at the basolateral side of the ileal cell by the ATP-driven exporter MRP1 or alternatively by a yet unknown mechanism. The vitamin remaining intracellularly is largely enzyme bound and serves as coenzyme for methionine synthase in the cytoplasm or for methylmalonyl CoA mutase in the mitochondria. Some Cbl may also be associated to the recently identified MMACHC protein involved in decyanation of CNCbl and the dealkylation of alkylcobalamins before conversion to coenzyme forms.^{35,36} In the plasma, free Cbl is immediately bound to transcobalamin (TC), synthesized and excreted in vesicles by the cells. (B) Nonpolarized cell expressing the TC receptor.

Mrp1^(-/-) mice^{23,33} have no major morphologic or metabolic abnormalities. Besides the change in Cbl homeostasis reported here, an increased sensitivity to some cytostatic drugs and altered inflammatory responses are the only abnormalities observed.^{23,33,34,41,47,48} Redundant drug/substrate export by one or other transporters may compensate for the lack of MRP1. An alternative efflux pathway of Cbl is actually suggested by our results because MRP1 deficiency did not prevent Cbl efflux completely. The role of MRP1 may be further defined by studying other species, including species in which it is possible to establish a diet-induced Cbl deficiency condition, to investigate whether failure of MRP-1 has a worsening effect.

The role of MRP1 in Cbl efflux of specific tissues and cell types merits further investigation. For instance, human Cbl deficiency is characterized by neurologic and hematologic symptoms, and the high level of MRP1 in the blood-cerebrospinal fluid barrier^{31,49} and red blood cells (see de Wolf et al⁵⁰) might make these tissues vulnerable to Cbl deficiency. MRP1 is also expressed at the basolateral surface of the syncytiotrophoblasts,⁵¹ but we did not find a role of MRP1 in fetal Cbl supply as the Cbl content of newborn offspring of the MRP1-deficient mice was not lowered (data not shown).

In conclusion, we have identified the ABC transporter MRP1 as the first known eukaryotic membrane efflux transporter capable of transporting free Cbl out of cells. Future studies may show other transporters assisting in the Cbl efflux and define whether polymorphisms or mutations in the genes encoding such transporters may affect the Cbl homeostasis in humans and contribute to Cbl deficiency disease. Moreover, the finding of MRP1-dependent Cbl

transport may influence on future strategies on cancer treatment with cytotoxic drugs in which MRP-1 function induces resistance to these drugs.

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Authorship

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