Heterogeneity in cblG: Differential retention of cobalamin on methionine synthase

ARTICLE *in* BIOCHEMICAL MEDICINE AND METABOLIC BIOLOGY · JULY 1992

DOI: 10.1016/0885-4505(92)90032-T · Source: PubMed

4 AUTHORS, INCLUDING:

Susan Sillaots

24 PUBLICATIONS **795** CITATIONS

SEE PROFILE

BIOCHEMICAL MEDICINE AND METABOLIC BIOLOGY 47, 242–249 (1992)

Heterogeneity in cblG: Differential Retention of Cobalamin on Methionine Synthase

S. L. SILLAOTS,* C. A. HALL,† V. HURTELOUP,* AND D. S. ROSENBLATT*

*MRC Genetics Group, Centre for Human Genetics, The Hess B. and Diane Finestone Laboratory in Memory of Jacob and Jenny Finestone and Departments of Medicine and Biology, McGill University, Montreal, Quebec, Canada H3A 1A1; and †Veterans Administration Medical Center, Albany, New York

Received February 10, 1992

Cultured fibroblasts from patients with functional methionine synthase deficiency have been shown to belong to two complementation classes, cblE and cblG. Both are associated with decreased intracellular levels of methylcobalamin (MeCbl) and decreased incorporation of label from 5-methyltetrahydrofolate into macromolecules. Methionine synthase specific activity is normal or near normal in cell extracts from cblE patients under standard reducing conditions, whereas specific activity is low in cblG extracts. Seven of 10 cblG cell lines accumulated [^{57}Co]CN-Cbl equivalent to control cells and showed similar proportions of label associated with the two intracellular cobalamin binders, methionine synthase and methylmalonyl-CoA mutase. The remaining three cblG lines showed reduced accumulation of labeled Cbl and virtually none associated with methionine synthase. The specific activity of methionine synthase was decreased in cell extracts from both cblG subgroups, being almost undetectable in extracts from the latter three lines. Incorporation of label from [^{14}C]MeTHF into either macromolecules or into methionine was decreased in both cblG groups, but was paradoxically higher in the three lines with very low *in vitro* methionine synthase activity. These results demonstrate further heterogeneity within cblG and suggest that the defect in the three variant lines affects the ability of methionine synthase to retain Cbl.

Inherited defects in vitamin B_{12} (cobalamin, Cbl) metabolism have been characterized by somatic cell complementation studies and defined alphabetically as cblA through cblG (1). Both cblE and cblG mutations are unable to synthesize methionine from homocysteine because of a functional defect in the cytoplasmic enzyme methionine synthase (5-methyltetrahydrofolate methyltransferase; EC 2.1.1.13). Methylcobalamin (MeCbl) acts as a cofactor for methionine synthase. Cultured fibroblasts from cblE and cblG patients do not grow in tissue culture medium in which homocysteine replaces methionine, have low incorporation of labeled methyltetrahydrofolate into macromolecules, and have low cellular levels of MeCbl.

Cbl, bound to the protein carrier transcobalamin II (TCII), is endocytosed into

0885-4505/92 $5.00

the mammalian cell and is digested free of the TCII. After Cbl exits from the lysosome to the cytoplasm, the cobalt core undergoes a reduction from Co^{3+} to Co^{2+}. At this point Cbl may bind to methionine synthase and be converted to MeCbl by transfer of the methyl group from *S*-adenosylmethionine (AdoMet). Subsequent methyl groups are donated to Co^{1+}-Cbl by 5-methyltetrahydrofolate. Cbl which does not bind to methionine synthase may enter the mitochondrion where reduction and adenosylation results in the formation of adenosylcobalamin (AdoCbl). AdoCbl acts as a cofactor for the conversion of methylmalonyl-CoA to succinyl-CoA, a mitochondrial reaction that is catalyzed by the enzyme methylmalonyl-CoA mutase (EC 5.4.99.2).

Clinically there is heterogeneity among both cblE and cblG patients (2–11). Most come to medical attention by the age of 2 years although one patient first appeared at age 21 (9). Both cblE and cblG patients exhibit homocystinuria and homocystinemia, megaloblastic anemia, and neurological defects, all of which respond to some extent to Cbl therapy (3).

The defect on cblE has been suggested to be in a reducing system associated with methionine synthase (12), whereas in cblG the defect seems to involve methionine synthase activity independent of the reducing system (2). Hall *et al.* (10) have proposed that the defect in cblG involves the way in which AdoMet interacts with methionine synthase.

We have examined the cellular distribution of label following incubation of cell lines from 7 cblE patients and 10 cblG patients with [^{57}Co]CN-Cbl. Our results indicate further heterogeneity within the cblG class.

MATERIALS AND METHODS

Cell Culture

Skin fibroblasts were obtained with informed consent from patients with MeCbl deficiency and included the cblE cell lines WG788, WG1146, WG1296, WG1384, WG1401, WG1575, and WB1663, and the cblG cell lines WG1308, WG1205, WG1223, WG1352, WG1386, WG1408, WG1505, WG1655, WG1670, and WG1671. Fibroblasts were stored at the Repository for Mutant Human Cells, Montreal Children's Hospital. All cell strains were determined to be free of mycoplasma contamination. Cultures were routinely maintained in Eagle's minimum essential medium plus nonessential amino acids (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS).

For studies of Cbl uptake and distribution, fibroblasts were incubated in either 25 or 50 pg/ml [^{57}Co]CN-Cbl as indicated.

Polyacrylamide Gel Electrophoresis

Fibroblasts were incubated in 50 pg/ml (37 pM) [^{57}Co]CN-Cbl + 10% human serum as a source of TC II for 5 days, trypsinized, and then resuspended in 600 or 800 μl of 0.25 M sucrose and 0.02 M Tris–HCl, pH 7.4. A small aliquot was removed for HPLC analysis of cobalamin distributions. The cell suspension was sonicated on ice, 3 × 30 s blasts at 12 μm, and the sonicate was centrifuged at 5°C at 245,000*g* for 30 min. The resulting supernatant was stored at −85°C until used for electrophoresis.

Supernatants were run in duplicate on a native polyacrylamide slab gel (4%) at pH 8.9. The gel was frozen, cut into 2-mm slices, and then analyzed in a scintillation counter (Beckman Instruments, Mississauga, Ontario).

Methionine Synthase Assay

The activity of methionine synthase activity was examined in cell extracts from the eight cblG lines that grew well enough to obtain sufficient cells for assay, and from six cblE lines. Confluent fibroblast cultures were trypsinized and resuspended at a density of 1–2 $\times$ 10^8 cells/ml in 0.25 M Sucrose. This cell suspension was sonicated on ice, 4 $\times$ 30 s blasts, and spun at 5°C at 170,000*g* for 45 min. The resulting supernatant was stored at −85°C. At the time of assay, the cell extract was diluted by 10% with a 1 M potassium phosphate buffer, pH 7.4, to form a cell extract in 0.1 M potassium phosphate buffer, pH 7.4

Enzyme activity was measured as previously described (2,13). The assay mixture for measurement of holoenzyme activity contained the following: varying amounts of cell extract and 0.1 M potassium phosphate for a total volume of 100 μl; 100 mM potassium phosphate buffer, pH 7.4; 250 μM *S*-adenosylmethionine; 500 μM D,L-homocysteine (prepared fresh daily from the thiolactone); 150 mM 2-mercaptoethanol; and 390 μM [^{14}C]methyltetrahydrofolate (1.4 dpm/pmol); for a total assay volume of 200 μl. For assay of total enzyme activity 50 μM of MeCbl was added.

The assay mixture was incubated in the dark at 37°C in stoppered vacutainer tubes that had been flushed with nitrogen for 7 s. The reaction was stopped with the addition of 800 μl of ice-cold distilled water, and the reaction mixture was applied to a syringe minicolumn with a bed volume of 1.5 ml Bio-Rad AG1X8, 200–400 mesh, chloride-form resin (Bio-Rad Laboratories). The reaction tubes were washed with another 1 ml of water which was applied to the columns. The resulting effluent of 2 ml was then analyzed for radioactivity in a Beckman scintillation counter.

Fractionation of Intracellular Cobalamins

Washed cell pellets of cells grown in 25 pg/ml of labeled cobalamin were extracted (14) in complete darkness in 10 ml of absolute ethanol at 85°C for 20 min and centrifuged, and 8 ml of supernatant ethanol was evaporated to dryness under a stream of nitrogen at room temperature. The remaining 1 ml aqueous sample was analyzed on high-pressure liquid chromatography using a Merck column Lichrosorb RP-8 (E. Merck, Darmstadt, Germany), 10 μm eluted with a gradient of phosphate at pH 3 and triethylammonium phosphate (15).

[^{14}C]Propionate and [^{14}C]Methyltetrahydrofolate Incorporation

The functional integrity of methylmalonyl-CoA mutase was assessed by measurement of incorporation of [^{14}C]propionate into acid-precipitable cellular macromolecules; the functional integrity of methionine synthase was assessed by measurement of the incorporation of label from [5-^{14}C]MeTHF into acid-precipitable macromolecules as previously described (3). The incorporation of label into

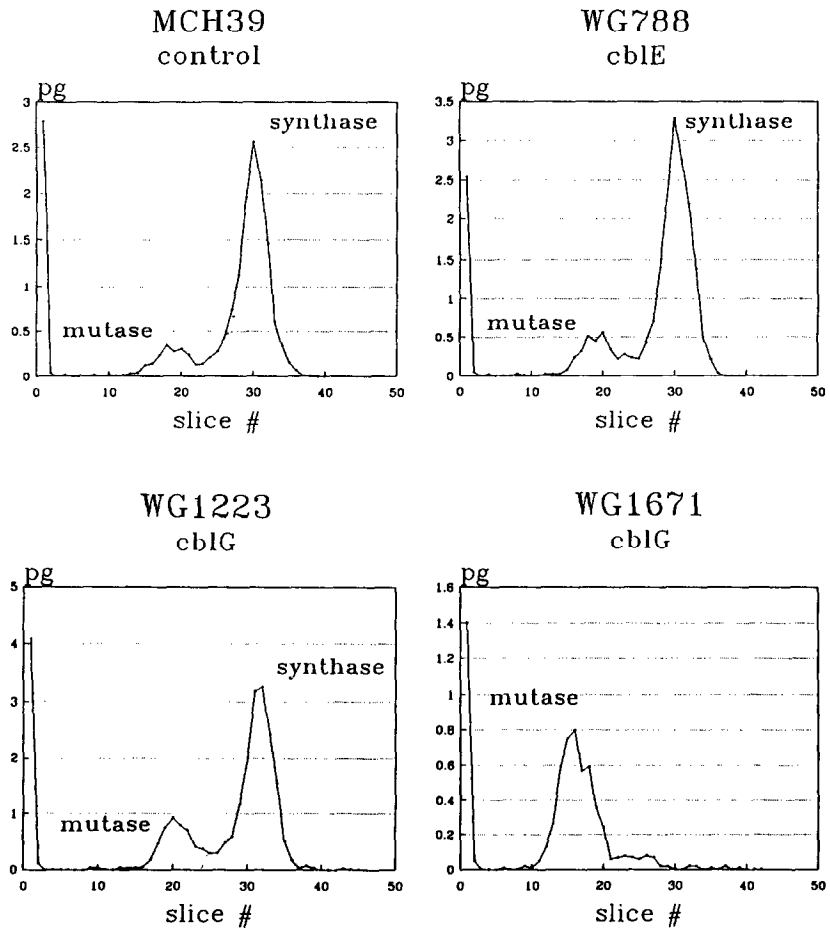

FIG. 1. [^{57}Co]CN-Cbl binding in fibroblast extracts from a control, a cblE, and two cblG patients after polyacrylamide gel electrophoresis. WG1671 represents one of three cblG cell lines which show no association of Cbl with methionine synthase.

methionine was determined following hydrolysis of cellular protein according to a modification of the method of Boss and Erbe (16).

Determination of Protein Content

Aliquots of cell extracts used for polyacrylamide gel electrophoresis and for methionine synthase assays were stored at −20°C until protein determinations were made according to the method of Lowry *et al.* (17). Bovine serum albumin (BSA) was used as a standard.

RESULTS

In control, cblE and cblG cell lines incubated for 5 days in 37 pM [^{57}Co]CN-Cbl, all label was found associated with the two Cbl-dependent enzymes. Two distinct distribution patterns were found (Fig. 1 and Table 1). In control, cblE,

TABLE 1
Enzyme-Bound Cobalamin

Cell line	Uptake (pg/mg protein)	% Mutase	% Synthase
Control (n = 3)	48.8 ± 4.2	15.7 ± 2.9	84.3 ± 2.9
cblE (n = 7)	52.1 ± 26.8	16.8 ± 3.6	83.2 ± 3.6
cblG (n = 7)	49.2 ± 18.3	25.5 ± 5.4	74.5 ± 5.4
cblG variant (n = 3)	29.9 ± 10.6	100	0

Note. [^{57}Co]CN-Cbl was preincubated with 10% human serum for 30 min at 37°C before incubation with cells. Cells were incubated for 5 days. The final CN-Cbl concentration was 37 pM. Results are shown as means ± standard deviations.

and 7 of 10 cblG lines, most of the label was found associated with methionine synthase and the remainder with methylmalonyl-CoA mutase. In three cblG lines, including those from a pair of siblings, virtually all of the accumulated label was found associated with the mutase enzyme. Total accumulation of enzyme-bound Cbl was lower in the three atypical of cblG lines than in the other groups but was higher than would be expected from lines that completely lacked methionine synthase.

The proportion of labeled CN-Cbl converted to MeCbl was decreased compared to that of controls in all cblE and cblG cell lines (Table 2). This was associated with increases in the other Cbl forms. In cblE lines and the seven cblG lines which had Cbl associated with methionine synthase there were increased proportions of Aq-Cbl, presumably reflecting Cbl intermediates on the enzyme. In addition the proportion of AdoCbl was increased. In the three cblG lines which had no Cbl associated with methionine synthase, there was a greater decrease in MeCbl, very little Aq-Cbl, and an even higher proportion of Cbl as AdoCbl.

TABLE 2
Cobalamin Distributions

	Aq-Cbl	CN-Cbl	AdoCbl	MeCbl	Others
Control	8.3	9.4	18.1	52.6	11.4
(n = 5)	(3.7)	(5.8)	(3.2)	(12.5)	(6.4)
cblE	24.2	19.0	23.2	11.4	21.1
(n = 7)	(9.2)	(15.2)	(8.0)	(6.8)	(6.1)
cblG	30.7	12.8	31.7	7.0	17.8
(n = 7)	(5.5)	(4.6)	(5.5)	(1.8)	(8.6)
cblG variant	3.8	10.3	62.9	3.5	19.7
(n = 3)	(3.2)	(2.9)	(9.7)	(2.0)	(7.8)

Note. Fibroblasts were incubated for 4 days at 2.5 pg/ml [^{57}Co]CN-Cbl. Extracts had protein removed and were then run on an HPLC in the dark as outlined under Methods. Abbreviations used: Aq, aquo; CN, cyano; Ado, adenosyl; Me, methyl. Standard deviations appear in parentheses.

TABLE 3
Methionine Synthase Activities in Fibroblast Extracts

	Methionine synthase activity (pmol methionine/min/mg protein)	
	Holoenzyme	Total enzyme
CblG (n = 5)	3.2 ± 1.5	9.3 ± 2.2
CblG variant (n = 3)	0.4 ± 0.4	0.5 ± 0.5
CblE (n = 6)	22.9 ± 5.1	77.3 ± 51.4
Control	23.3 ± 12.5	75.4 ± 31.9

Note. Cells were grown in the absence of OHCbl, extracts were prepared, and methionine synthase activity was determined in the extracts as per the method of Watkins and Rosenblatt (2). Holoenzyme represents activity without adding MeCbl to the reaction, whereas total enzyme represents activity in the presence of MeCbl. CblG variant represents the three cblG cell lines that show no association of Cbl with methionine synthase.

The uptake of propionate was equivalent to control values in all cblG cell lines. The uptake of methyltetrahydrofolate was low in all cblG lines. For the seven cblG cell lines that bound Cbl to methionine synthase, MeTHF uptake was 29.8 pmol/mg protein (±17.4) for cells grown in 0.75 μM OH-Cbl, and 22.2 pmol/mg protein (±14.3) for cells not grown in Cbl. In the three atypical lines, the methyl uptake was 50.0 pmol/mg protein (±10.8) and 46.0 pmol/mg protein (±7.0), respectively. Control uptakes of MeTHF were 185.3 pmol/mg protein (±57.3) and 100.22 pmol/mg protein (±43.26). The three atypical lines had significantly higher MeTHF uptake than the classical cblG lines for cells grown in Cbl (Student t test, $P < 0.01$). Studies on the amount of label incorporated into methionine for cells grown in medium containing Cbl were 82.9 ± 16.4 pmol/mg protein (n = 3, controls), 15.5 ± 18.6 pmol/mg protein (n = 3, classical cblG), and 22 ± 6.8 pmol/mg protein (n = 3, cblG variant).

Methionine synthase specific activities from cells at confluence grown in medium without OH-Cbl supplementation and assayed under standard reducing conditions are shown in Table 3. Under these conditions all cblG cell lines have low activity but the three atypical cblG lines showed activities very close to background levels.

DISCUSSION

Patients with cblG disease usually come to medical attention by the age of 2 years with varying degrees of homocystinuria, homocystinemia, hypomethioninemia, megaloblastic anemia, and neurological deficits (3). However, one cblG patient presented in adulthood with ataxia and was misdiagnosed as having multiple sclerosis (9).

Fibroblasts from these patients tend to exhibit normal propionate incorporation and low methyltetrahydrofolate incorporation. They also exhibit normal AdoCbl levels and low MeCbl levels. Although most cblG cells have demonstrated low

methionine synthase activities under standard reducing conditions, some cell lines have demonstrated normal activities, further illustrating the heterogeneity within this group (10,11). Tissue culture complementation studies have shown that all cblG cell lines appear to belong to the same complementation class.

In this study, we have examined the pattern of Cbl association with the two Cbl-dependent enzymes in cells from patients with cblG disease and found heterogeneity among the cell lines. Seven out of 10 cblG cell lines exhibited a pattern similar to that seen in control cells with roughly 15% of the label attached to the mutase enzyme and the remaining 85% attached to the methionine synthase enzyme. In three cblG cell lines, two of which are from siblings, there was no Cbl associated with methionine synthase. In these three cell lines, there was a lowered incorporation of labeled Cbl, but it was still at a relatively high level. Since control cells bind roughly 85% of intracellular cobalamin to methionine synthase, and all intracellular cobalamin is found bound to either the mutase or synthase enzymes, one would expect that a mutant which demonstrates a lack of association of Cbl to methionine synthase would show an 85% decrease in Cbl uptake. This has not been observed. It is possible that most of the excess cobalamin leaves the cytoplasm and enters the mitochondria where it would be utilized by methylmalonyl-CoA mutase.

The patients whose cell lines showed no Cbl associated with the synthase enzyme all had severe clinical manifestations presenting at an early age. All three remain developmentally delayed despite treatment with Cbl.

The methionine synthase specific activities in the three atypical lines was almost undetectable. However, the three atypical lines had levels of MeTHF incorporation into methionine that were detectable, and indeed higher than those seen in the lines that showed Cbl associated with methionine synthase. These findings suggest that some methionine synthase is present in all cblG cell lines. All the cblG cell lines fall into the same complementation class. It has been suggested previously that the defect in some of the cblG patients may relate to the interaction of AdoMet with methionine synthase (10). The three atypical patients described have a defect which affects the recovery of Cbl on methionine synthase.

ACKNOWLEDGMENTS

Cell lines described in this report were kindly provided by S. Schmutz (University of Saskatchewan, Winnepeg), R. Erbe (Harvard Medical School, Boston), H. Morton (The Children's Hospital of Philadelphia), C. Krumdieck (University of Alabama at Birmingham), R. Schutgens (University Hospital, Amsterdam), D. B. Flannery (Medical College of Georgia, Augusta), H. L. Levy (The Children's Hospital, Boston), Y. S. Shin (University of Munich), R. Carmel (USC School of Medicine, Los Angeles), and E. Roback (Vanderbilt University, Nashville). The authors thank Nora Matiaszuk for tissue culture and substrate uptake studies, Helena Leu-Shing for HPLC analysis, Angela Hosack for technical assistance, and David Watkins for methionine synthase assay data. Bernard A. Cooper and Bernadette Chadefaux provided helpful discussion and technical advice.

REFERENCES

1. Cooper BA, Rosenblatt DS. Inherited defects of vitamin B_{12} metabolism. *Annu Rev Nutr* **7**:291–320, 1987.
2. Watkins D, Rosenblatt DS. Genetic heterogeneity among patients with methylcobalamin deficiency. *J Clin Invest* **81**:1690–1694, 1988.

3. Watkins D, Rosenblatt DS. Functional methionine synthase deficiency (cblE and cblG): clinical and biochemical heterogeneity. *Am J Med Genet* **34:**427–434, 1989.
4. Rosenblatt DS, Cooper BA, Schmutz SM, Zaleski WA, Casey RE. Prenatal vitamin B_{12} therapy of a fetus with methylcobalamin deficiency (cblE disease). *Lancet* **1:**1127–1129, 1985.
5. Rosenblatt DS, Thomas IT, Watkins D, Cooper BA, Erbe RW. Vitamin B_{12} responsive homocystinuria and megaloblastic anemia: heterogeneity in methylCbl deficiency. *Am J Med Genet* **26:**377–383, 1987.
6. Schuh S, Rosenblatt DS, Cooper BA, Schroeder ML, Bishop AJ, Seargeant LE, Haworth JC. Homocystinuria and megaloblastic anemia responsive to vitamin B_{12} therapy. *N Engl J Med* **310:**686–690, 1984.
7. Shin YS, Reiter S, Zelger O, Brunstler I, Rucher AV. Orotic aciduria, homocystinuria, formiminoglutamic aciduria and megaloblastosis associated with the formiminotransferase/cyclodeaminase deficiency. In Purine and Pyrimidine Metabolism in Man (Nyhan WL, Thompson F, Watts RWE, Eds.). New York: Plenum, 1986, pp 71–76.
8. Tuchman M, Kelly P, Watkins D, Rosenblatt DS. Vitamin B_{12}-responsive megaloblastic anemia, homocystinuria and transient methylmalonic aciduria in cblE disease. *J Pediatr* **113:**1052–1056, 1988.
9. Carmel R, Watkins D, Goodman SI, Rosenblatt DS. Hereditary defect of cobalamin metabolism (cblG mutation) presenting as a neurological disorder in adulthood. *N Engl J Med* **318:**1738–1741, 1988.
10. Hall CA, Lindenbaum RH, Arenson E, Begley JA, Chu RC. The nature of the defect in cbl-G mutation. *Clin Invest Med* **12:**262–269, 1989.
11. Rosenblatt DS, Cooper BA. Selective deficiencies of methyl-B_{12} (cblE and cblG). *Clin Invest Med* **12:**270–271, 1989.
12. Rosenblatt DS, Cooper BA, Pottier A, Lue-Shing H, Matiaszuk N, Grauer K. Altered vitamin B_{12} metabolism in fibroblasts from a patient with megaloblastic anemia and homocystinuria due to a new defect in methionine biosynthesis. *J Clin Invest* **74:**2149–2156, 1984.
13. Mellman I, Willard HF, Rosenberg LE. Cobalamin binding and cobalamin dependent enzyme activity in normal and mutant human fibroblasts. *J Clin Invest* **62:**952–960, 1978.
14. Linnell JC, MacKenzie HM, Wilson J, Matthews DM. Patterns of plasma cobalamins in control subjects and in cases of vitamin B_{12} deficiency. *J Clin Pathol* **22:**545–550, 1969.
15. Jacobsen D, Green WR, Quadros EV, Montejado YD. Rapid analysis of cobalamin coenzymes and related corrinoid analogs by high-performance liquid chromatography. *Anal Biochem* **120:**394–403, 1982.
16. Boss GR, Erbe RW. Decreased rates of methionine synthesis by methylenetetrahydrofolate reductase-deficient fibroblasts and lymphoblasts. *J Clin Invest* **67:**1659, 1981.
17. Lowry OM, Rosenbrough NJ, Farr AL. Protein measurement with the folin phenol reagent. *J Biol Chem* **193:**265–275, 1951.