Complementation studies in the *cblA* class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (*cblH*)

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Abstract

Aim—To investigate genetic heterogeneity within the *cblA* class of inborn error of cobalamin metabolism.

Context—The *cblA* disorder is characterised by vitamin B12 (cobalamin) responsive methylmalonic aciduria and deficient synthesis of adenosylcobalamin, required for activity of the mitochondrial enzyme methylmalonyl CoA mutase. The *cblA* gene has not been identified or cloned. We have previously described a patient with the clinical and biochemical phenotype of the *cblA* disorder whose fibroblasts complemented cells from patients with all known types of inborn error of adenosylcobalamin synthesis, including *cblA*.

Methods—We have performed somatic cell complementation analysis of the *cblA* variant fibroblast line with a panel of 28 *cblA* lines. We have also performed detailed complementation analysis on a panel of 10 *cblA* fibroblast lines, not including the *cblA* variant line.

Results—The *cblA* variant line complemented all 28 cell lines of the panel. There was evidence for interallelic complementation among the 10 *cblA* lines used for detailed complementation analysis; no cell line in this panel complemented all other members.

Conclusions—These results strongly suggest that the *cblA* variant represents a novel complementation class, which we have designated *cblH* and which represents a mutation at a distinct gene. They also suggest that the *cblA* gene encodes a protein that functions as a multimer, allowing for extensive interallelic complementation.

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Methylmalonyl CoA mutase is one of two enzymes in mammalian cells that require derivatives of cobalamin (vitamin B12) for activity. This mitochondrial enzyme catalyses the conversion of methylmalonyl CoA, generated during catabolism of branched chain amino acids and odd chain fatty acids, to succinyl CoA which can enter the tricarboxylic acid cycle.¹ A number of inborn errors of metabolism have been identified that result in decreased methylmalonyl CoA mutase activity,

either as the result of mutations affecting the gene encoding the enzyme itself (the *mut* class of mutations) or as the result of mutations that cause decreased synthesis of the adenosylcobalamin (AdoCbl) cofactor required for its activity (*cblA*, *cblB*, *cblC*, *cblD*, and *cblF*). Patients with decreased synthesis of AdoCbl present with cobalamin responsive methylmalonic aciduria.

Synthesis of AdoCbl from exogenous cobalamin involves reduction of the central cobalt atom of the cobalamin molecule followed by reaction of the fully reduced cobalamin with ATP.^{2–3} This process is disrupted in cells from patients with the *cblA* and *cblB* disorders. Synthesis of AdoCbl from exogenous cobalamin and activity of methylmalonyl CoA mutase are decreased in *cblA* and *cblB* fibroblasts; activity of the second cobalamin dependent enzyme, methionine synthase, and synthesis of its methylcobalamin (MeCbl) coenzyme are normal. Synthesis of AdoCbl from exogenous cobalamin occurs in extracts of fibroblasts from patients with the *cblA* disorder in the presence of chemical reducing agents, but not in *cblB* fibroblast extracts under the same conditions.^{4–5} It has thus been concluded that mutations in the *cblB* disorder affect the gene encoding cobalamin adenosyltransferase, while *cblA* mutations affect a cobalamin reductase. Neither the *cblA* nor the *cblB* gene has been cloned. The *cblA* and *cblB* disorders can be differentiated on the basis of complementation analysis using cultured fibroblasts. The classification of patients with these disorders is of clinical importance because, as a group, *cblA* patients have a better prognosis than do *cblB* patients.⁶

We have previously described a patient with clinical and biochemical features of the *cblA* disorder, including ability of cell extracts to synthesise AdoCbl from exogenous cobalamin, whose fibroblasts complemented with other *cblA* fibroblast lines as well as fibroblasts from all other classes of inborn errors resulting in methylmalonic aciduria.⁷ These results suggested that this patient might represent a previously unrecognised complementation class. However, because it was tested against only a small number of *cblA* cell lines, it was not possible to be certain that this was not an instance of interallelic complementation, and the patient has therefore been classified as a *cblA* variant. We have now conducted complementation analysis on a large panel of *cblA* fibroblast lines, including the *cblA* variant line,

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to determine whether interallelic complementation occurs among *cblA* cell lines, and whether the *cblA* variant cell line represents a novel inborn error of cobalamin metabolism.

Materials and methods

CELL CULTURE

Complementation analysis was performed using a panel of 29 *cblA* fibroblast lines, including the *cblA* variant cell line WG1437, which was tested against all other members of the panel. Detailed complementation analysis was carried out on a smaller subpanel of 10 *cblA* fibroblast lines; all possible pairwise combinations were tested in this subpanel. The cell lines were sent to the laboratory in the Division of Medical Genetics at the Royal Victoria Hospital in Montreal for diagnosis and were from patients with clinical methylmalonic aciduria. All cell lines were tested for ability to synthesise cobalamin coenzyme derivatives from [⁵⁷Co]CN Cbl, and incorporation of label from [1-¹⁴C]propionate into cellular macromolecules (a measure of function of methylmalonyl CoA mutase in intact cells). Fibroblasts from *cblA* patients typically showed reduced synthesis of AdoCbl and decreased propionate incorporation, in the presence of normal MeCbl synthesis. The diagnosis of *cblA* disorder in all cell lines, with the exception of the *cblA* variant cell line WG1437, was established by complementation analysis against a panel of fibroblasts from patients with confirmed diagnosis of various classes of inborn error of cobalamin metabolism resulting in methylmalonic aciduria. *cblA* fibroblasts complemented authentic *cblB*, *cblC*, and *cblD* fibroblasts, but did not complement *cblA* fibroblasts.

Cells were routinely maintained in minimum essential medium plus non-essential amino acids (MEM, Gibco) supplemented with 5% fetal bovine serum (Intergen) and 5% iron enriched calf serum (Intergen).

PROPIONATE INCORPORATION

Activity of methylmalonyl CoA mutase was assessed indirectly by measurement of incorporation of label from [1-¹⁴C]propionate into cellular macromolecules.⁸ Cultures were incubated for 18 hours in Puck's F medium supplemented with 15% fetal bovine serum (Intergen) and 100 µmol/l [1-¹⁴C]propionate (New England Nuclear; diluted with unlabelled propionate to give a final specific activity of 10 µCi/µmol). At the end of this incubation, cellular macromolecules were precipitated with 5% trichloroacetic acid (TCA). The precipitated material was dissolved in 0.2 N sodium hydroxide and radioactivity was determined by liquid scintillation counting.

COMPLEMENTATION ANALYSIS

Equal numbers of two cell lines were plated together in 35 mm tissue culture dishes at a total density of 400 000 cells per dish. Twenty four hours after plating, half of the cultures were fused by exposure for 60 seconds to a 40% (v/v) solution of polyethylene glycol 1000 (PEG, J T Baker Inc) in phosphate buffered saline. Cultures were washed thoroughly using

tissue culture medium lacking serum to remove any residual PEG. Propionate incorporation in heterokaryons was determined 24 hours after fusion.⁹ Complementation was considered to have occurred when propionate incorporation was significantly greater in fused mixed cultures of two cell lines than in parallel unfused mixed cultures, as determined by Student's *t* test ($p < 0.01$). As well, propionate incorporation in mixed unfused cultures was compared with values for unmixed cultures of the two cell lines to determine whether there was evidence for correction in the absence of fusion.

Results

Complementation is typically detected by comparison of parallel fused and unfused cultures; complementation occurs when propionate incorporation is significantly greater in mixed, fused cultures of two cell lines than in parallel mixed, unfused cultures. Mixture of two *cblA* fibroblast lines in the absence of PEG typically results in levels of propionate incorporation intermediate between those of the two cell lines incubated alone. However, in certain combinations of *cblA* fibroblasts, propionate incorporation was observed to be increased to levels greater than that of either line alone in mixed cultures that had not been exposed to PEG (data not shown). In the present study, complementation was considered to occur if propionate incorporation was significantly greater in fused mixed cultures of two cell lines than in parallel unfused mixed cultures, or if propionate incorporation was significantly increased in mixed unfused cultures of the two cell lines compared to the average of the two cell lines incubated separately, even if incorporation did not further increase in mixed fused cultures.

The *cblA* variant line WG1437 was tested against the entire panel of 28 *cblA* fibroblast lines (table 1). In all cases, propionate incorporation was significantly greater in mixed fused cultures of the two cell lines than in parallel mixed unfused cultures ($p < 0.01$ by Student's *t* test). Thus, WG1437 complemented all other *cblA* cell lines in the panel.

Detailed complementation analysis was carried out on a panel of 10 *cblA* fibroblast lines. Results of these studies are summarised in fig 1. Several pairs of *cblA* cell lines were found to complement one another, although no cell line complemented all other members of the panel. Certain cell lines, such as WG2278, frequently complemented other *cblA* lines, while others complemented less frequently. WG1191 and WG1518 did not complement any member of the panel.

Discussion

Synthesis of the AdoCbl coenzyme required for activity of methylmalonyl CoA mutase in mammalian cells involves reduction of the central cobalt atom of cobalamin followed by reaction with ATP. Reduction is believed to be a two step process, with reduction of cob(III)alamin to cob(II)alamin and of cob(II)alamin to cob(I)alamin catalysed by separate enzymes, by analogy with bacteria.^{2,3} Only the fully reduced

Table 1 Complementation analysis of *cblA* variant line WG1437 with *cblA* fibroblast lines

Cell line	Propionate incorporation (nmol propionate incorporated/mg protein/18 h)			
	WG1437 alone	Cell line alone	Mixed unfused	Mixed fused
WG1191	0.15 ± 0.01	1.08 ± 0.11	0.62 ± 0.13	3.69 ± 1.02
WG1192	0.94 ± 0.03	1.26 ± 0.10	1.12 ± 0.07	3.24 ± 0.14
WG1411	0.86 ± 0.04	1.23 ± 0.15	1.13 ± 0.03	2.23 ± 0.20
WG1449	0.55 ± 0.13	1.05 ± 0.04	0.66 ± 0.01	2.44 ± 0.10
WG1516	0.79 ± 0.10	2.49 ± 0.10	0.90 ± 0.03	2.61 ± 0.10
WG1588	0.67 ± 0.06	1.49 ± 0.01	0.96 ± 0.03	3.21 ± 0.25
WG1660	0.86 ± 0.15	0.82 ± 0.94	1.04 ± 0.06	1.60 ± 0.09
WG1776	0.54 ± 0.01	0.90 ± 0.04	0.96 ± 0.07	2.37 ± 0.08
WG1796	0.86 ± 0.04	1.11 ± 0.15	1.28 ± 0.07	2.34 ± 0.10
WG1798	0.91 ± 0.03	3.46 ± 0.08	2.77 ± 0.11	4.08 ± 0.14
WG1802	0.80 ± 0.23	1.20 ± 0.07	0.99 ± 0.02	2.41 ± 0.33
WG1943	0.67 ± 0.02	1.47 ± 0.07	0.96 ± 0.02	3.54 ± 0.29
WG1997	0.55 ± 0.07	2.90 ± 0.13	2.37 ± 0.01	3.76 ± 0.10
WG2014	0.73 ± 0.05	1.91 ± 0.03	1.82 ± 0.14	7.27 ± 0.81
WG2019	2.17 ± 0.12	1.40 ± 0.12	1.30 ± 0.10	2.47 ± 0.29
WG2037	2.17 ± 0.12	1.67 ± 0.12	1.27 ± 0.15	2.50 ± 0.10
WG2063	0.73 ± 0.05	1.73 ± 0.03	0.84 ± 0.03	2.30 ± 0.16
WG2185	0.79 ± 0.06	1.96 ± 0.03	0.98 ± 0.08	2.52 ± 0.12
WG2188	0.55 ± 0.13	0.97 ± 0.03	0.82 ± 0.16	2.67 ± 0.12
WG2230	0.54 ± 0.01	1.64 ± 0.14	0.88 ± 0.02	3.27 ± 0.09
WG2278	0.74 ± 0.16	4.07 ± 0.17	2.48 ± 0.13	5.30 ± 0.41
WG2326	1.13 ± 0.12	0.73 ± 0.09	0.66 ± 0.09	3.67 ± 0.15
WG2529	0.73 ± 0.05	1.12 ± 0.22	0.81 ± 0.01	3.78 ± 0.13
WG2578	0.46 ± 0.07	0.83 ± 0.05	0.66 ± 0.01	1.41 ± 0.09
WG2623	0.55 ± 0.13	0.48 ± 0.03	0.60 ± 0.02	1.54 ± 0.10
WG2627	0.70 ± 0.17	1.44 ± 0.13	0.79 ± 0.04	5.33 ± 0.19
WG2653	0.20 ± 0.04	0.62 ± 0.02	0.52 ± 0.13	3.37 ± 0.76
WG2664	0.70 ± 0.17	1.77 ± 0.22	0.98 ± 0.01	4.00 ± 0.29

Propionate incorporation was measured in unmixed cultures of WG1437 and the *cblA* line tested, in mixed cultures of the two cell lines that had not been exposed to PEG (mixed unfused), and in mixed cultures that had been fused by exposure to 40% PEG. Propionate incorporation was significantly increased ($p < 0.01$ by Student's *t* test) in mixed fused cultures compared to mixed unfused cultures in all cases.

cob(I)alamin species can serve as substrate in cobalamin adenosylation. The enzymes catalysing these steps have not been identified or characterised, however, and the genes encoding them have not been cloned. A number of proteins with cobalamin reductase activity have been isolated from mammalian cells,^{10–14} but it is not clear whether these reduce cobalamin under physiological conditions. A mitochond-

drial NADPH linked aquacobalamin reductase has been reported to be deficient in a single *cblA* fibroblast line (WG2230),¹⁵ but this result has not been tested in additional *cblA* lines.

Somatic cell complementation analysis has been extensively used in classification of inborn errors of cobalamin metabolism. To date, seven complementation classes of mutants affecting cobalamin metabolism, designated *cblA*–*cblG*, have been identified. Of these, the *cblA* and *cblB* classes affect synthesis of AdoCbl alone, the *cblC*, *cblD*, and *cblF* classes affect synthesis of both AdoCbl and MeCbl, and thus affect earlier steps in cobalamin metabolism common to the synthesis of both coenzyme derivatives, while the *cblE* and *cblG* classes affect synthesis of MeCbl alone. Different complementation classes represent mutations at separate loci. However, interallelic complementation can occur within complementation classes under certain circumstances, for example at loci coding for multimeric proteins. Interallelic complementation has been observed in mutations affecting several human genes, including those encoding methylmalonyl CoA mutase,¹⁶ propionyl CoA carboxylase,¹⁷ and argininosuccinate lyase.¹⁸

The existence of the *cblA* variant patient whose fibroblasts complement other *cblA* fibroblast lines⁷ indicates that there is heterogeneity among patients with the *cblA* phenotype, although it has not been clear whether this heterogeneity represents interallelic or intergenic complementation. The results of the present study show that heterogeneity exists at both levels among patients that have been assigned to the *cblA* complementation class.

The *cblA* variant fibroblast line WG1437 was found to complement each of the other 28 cell

Figure 1 Complementation among *cblA* fibroblast lines. Ability of pairs of *cblA* fibroblast lines to complement one another is shown. The difference between propionate incorporation (nmol propionate/mg protein/h) in parallel fused and unfused mixed cultures of the two cell lines; a positive value indicates that incorporation increased following fusion. Shaded squares indicate pairs of *cblA* lines that complement one another ($p < 0.01$); open squares indicate pairs of *cblA* lines that do not complement one another.

lines in the *cblA* panel (table 1). This strongly suggests that WG1437 represents a novel complementation class separate from the *cblA* class, and that the mutation in this patient affects a gene different from the gene affected in *cblA* patients. The designation *cblH* is suggested for this group.

Complementation was also observed among fibroblast lines from patients with the *cblA* disorder. No cell line among the panel in the present study complemented all other members of the class, and the pattern of complementation observed among these lines is most consistent with interallelic complementation (fig 1). Fibroblast lines WG1191 and WG1518 appear to define the *cblA* class, since they do not complement any other member of the panel. Existence of interallelic complementation among *cblA* fibroblast lines suggests that the product of the *cblA* gene functions as a multimer of some type or as part of a multi-subunit enzyme containing more than one copy of the *cblA* gene product.

The identities of the *cblA* and *cblH* gene products are unknown. The defect in both classes of mutation results in decreased synthesis of the cob(I)alamin substrate required for synthesis of AdoCbl, and can be bypassed in cell free extracts by provision of exogenous reducing agent. The two gene products might represent separate enzymes acting on cobalamin or might represent subunits of a single protein. Until the genes and gene products are identified, it is not possible to distinguish between these possibilities.

The existence of interallelic complementation within the *cblA* means that care must be taken in using complementation analysis to establish the diagnosis of patients referred for methylmalonic aciduria. Use of cell lines characterised by little or no interallelic complementation, such as WG1191 and WG1518 in the present panel, should minimise the possibility of false diagnosis.

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