Disruption of a Regulatory System Involving Cobalamin Distribution and Function in a Methionine-dependent Human Glioma Cell Line*

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Cobalamin metabolism and function were investigated at the levels from transcobalamin II (TCII) receptor to the cobalamin-dependent enzymes, methionine synthase and methylmalonyl-CoA mutase, in a methionine-dependent (P60) and a methionine-independent (P60H) glioma cell line. Using P60H as reference, the P60 cells cultured in a methionine medium had slightly lower TCII receptor activity and normal total cobalamin content, a moderately reduced microsomal and mitochondrial cobalamin(III) reductase activity but only trace amounts of the methylcobalamin and adenosylcobalamin cofactors. When transferred to a homocysteine medium without methionine, P60H cells showed a slightly enhanced TCII receptor activity, but the other cobalamin-related functions were essentially unchanged. In contrast, the methionine-dependent P60 cells responded to homocysteine medium with a nearly 6-fold enhancement of TCII receptor expression and a doubling of both the hydroxycobalamin content and the microsomal reductase activity. The mitochondrial reductase and the cobalamin-related processes further down the pathway did not change markedly. In both cell lines, TCII receptor activity was further increased when growth in homocysteine medium was combined with N_2O exposure.

These data suggest that low methionine and/or high homocysteine exert a positive feedback control on TCII receptor activity. The concurrent increase in hydroxycobalamin content and in microsomal reductase activity are either subjected to similar regulation or secondary to increased cobalamin transport. This regulatory network is most prominent in the methionine-dependent P60 cells harboring a disruption of the network in the proximity of cobalamin(III) reductase.

Disturbance of cellular Met metabolism is a common feature of cancer cells. This trait, known as Met dependence, becomes apparent when cells are cultured in a medium where Met is replaced with homocysteine (Hcy).[1] Met-dependent cells cease

to proliferate, whereas the Met-independent cells thrive under such conditions (1).

The biochemical basis for Met dependence is related to an imbalance between Met consumption and formation. In most cells, Met is formed from Hcy in a reaction catalyzed by 5-methyltetrahydrofolate homocysteine methyltransferase (Met synthase, EC 2.1.1.13.). This enzyme uses methyltetrahydrofolate (CH_3THF) as substrate, and methylcobalamin (CH_3Cbl) serves as cofactor (2). A common finding in the Met-dependent cells is a low rate of Hcy remethylation (1), which seems related to lack of CH_3Cbl (3, 4). Certain inborn errors in cobalamin (Cbl) metabolism (5), Cbl deficiency as well as N_2O exposure, are associated with marked reduction in Hcy remethylation and may render the cells fully or partly dependent on an exogenous supply of Met. Thus, these clinical conditions may have biochemical characteristics similar to those observed in Met-dependent cancer cells.

Cellular Cbl metabolism is only partly characterized (Fig. 1). Cbl crosses the cell membrane as a complex with transcobalamin II (TCII). This transport is mediated by a specific TCII receptor (6). In the lysosomes, Cbl is released from this complex (5), followed by an axial ligand exchange (7, 8). The Cbl(III) then becomes reduced to Cbl(II) (9), probably by a NADH-linked Cbl(III) reductase (CR) located either in the microsomes or in the mitochondrial membrane (8, 10). The Cbl(II) thus formed becomes bound to the cytosolic Met synthase. Alternatively, it undergoes an additional reduction in the mitochondria, probably catalyzed by a NADPH-dependent enzyme (10), to Cbl(I), followed by conversion to adenosylcobalamin (AdoCbl) (11). This Cbl species serves as the cofactor for methylmalonyl-CoA mutase (MCM) (EC 5.4.99.2), which is localized in the mitochondrial matrix and catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA (2, 12).

We have previously (3, 13) shown that the Met-dependent variant of a human glioma cell line is related to a biochemical defect leading to low levels of both CH_3Cbl and AdoCbl, thus resembling the inborn errors of Cbl metabolism belonging to the complementation classes C and D (5). In the present study, we investigated key processes operating at different levels along the Cbl pathway, from transport to metabolism to the catalytic function of the Cbl-dependent enzymes. These processes were studied in the Met-dependent P60 and the Met-independent P60H variants of a human glioma cell line, cultured in a Met medium and after transfer to a medium lacking

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[1] The abbreviations used are: Hcy, homocysteine; CH_3THF, methyltetrahydrofolate; CH_3Cbl, methylcobalamin; Cbl(s), cobalamin(s); TCII,

transcobalamin II; CR, cobalamin(III) reductase; AdoCbl, adenosylcobalamin; MCM, methylmalonyl-CoA mutase; CNCbl, cyanocobalamin; OHCbl, hydroxycobalamin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; BAEE, benzoyl-L-arginine ethyl ester units.

Met but supplemented with Hcy, and also during N_2O exposure which irreversibly inactivates Met synthase (14). The results point to the site of metabolic change responsible for the Met-dependent phenotype and also unravel the dynamics of a Cbl regulatory network under conditions of metabolic stress.

MATERIALS AND METHODS
Chemicals

DL-Methylmalonyl-CoA (lithium salt), succinyl-CoA (sodium salt), 5′-deoxyAdoCbl, cyanoCbl (CNCbl), folic acid, L-Met, thymidine, hypoxanthine, bovine serum albumin (deficient in Cbl and Cbl-binding proteins), bovine serum albumin (fatty acid-free), leupeptin (hemisulfate salt), trypsin inhibitor, pepstatin A, phenylmethylsulfonyl fluoride, Trizma (Tris base), and NADH (disodium salt) were from Sigma. Aprotinin, hydroxyCbl (OHCbl), L-Hcy thiolactone, and bis(3,5,5-trimethylhexyl)phthalate were from Fluka Chemie AG (Buchs, Switzerland). 2-Mercaptoethanol (pro analysis) and polyvinylpyrrolidone were from Merck (Darmstadt, Germany). BCA protein assay reagent was purchased from Pierce and Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies, Inc. (Paisley, Scotland).

Cells and Culture Conditions

The two cell lines, the Met-dependent P60 and the Met-independent P60H, have been described previously (3, 13). They are both variants of the GaMg cell line, established from a human glioblastoma multiform tumor in a 42-year-old female (15). Their different abilities to utilize Hcy for growth were developed by culture for about 50 passages in a medium containing Met (P60) or a medium in which Hcy replaced Met (P60H) (13).

Stem cultures were kept in DMEM supplemented with 330 μM non-essential amino acids, 0.6 g/liter L-glutamine, 1.5 μM CNCbl, 10 μM folic acid, 5 μM thymidine, 40 μM hypoxanthine, and 10% heat-inactivated fetal calf serum. P60 was kept in a medium containing 50 μM Met (Met medium) whereas P60H was kept in a Met-free medium supplemented with 200 μM Hcy thiolactone (Hcy medium). P60H cells were grown one passage in Met medium prior to the start of experiments. The cells were cultured in an atmosphere of 5% CO_2, 95% air at 37 °C with relative humidity of 98%.

Experimental Design

Cells were always seeded in Met medium, at a density of approximately 1,000 cells/cm^2 and 10,000 cells/ml. For most experiments, we used 10-cm dishes. T175 flasks were used for CR, titanium citrate, and NADPH assay. 6-cm dishes were used for conventional assay of Met synthase. To reach early logarithmic growth phase, P60 cells were incubated for 3 days and P60H for 4 days. The medium was then changed to either Met medium or Hcy medium. The cells were allowed to incubate with the fresh medium for 3 h, and they were then placed in modular incubation chambers (Billups-Rothenberg, Del Mar, CA) and exposed to either air or N_2O for 48 h (3). The cells were in late exponential growth phase when harvested.

Dishes for measurement of TCII receptor activity were harvested by use of a rubber policeman after removing the medium and adding 3 ml of phosphate-buffered saline (PBS). The suspension was centrifuged, resuspended in 1 ml of PBS, and stored at −80 °C until analysis.

Dishes for the other assays were trypsinized (0.1 mg/ml, BioWhittaker, Gaithersburg, MD). Samples for analysis of Cbl were washed with PBS-containing albumin (0.1 mg/ml), and the final pellet was resuspended in 300 μl of this solution and frozen at −80 °C. Samples for analysis of MCM were resuspended in 300 μl of 20% glycerol and frozen at −80 °C. Samples for analysis of CR were washed with PBS-containing trypsin inhibitor (10 BAEE units/ml), resuspended in isolation buffer (see below), and then placed on ice, and the activity was determined the same day.

Analytical Procedures

Cobalamin—The details of the sample preparation for analysis of Cbl has been published before (3, 13). Cbl was extracted by heating in the presence of acetic acid and *N*-ethylmaleimide. Separation of the different forms of Cbl was performed by reversed-phase HPLC (16). The retention times for the Cbl forms were: 10.5 min (OHCbl), 12 min (CNCbl), 14 min (AdoCbl), and 16 min (CH$_3$Cbl). Total Cbl and Cbl in the various fractions were determined by a radioimmunodilution assay (17). Salivary R-binder was used as binding protein.

TCII Receptor Binding Activity—The assays were performed according to Seligman and Allen (18) by the DEAE-Sephadex method using 2 pmol of human TCII-[^{57}Co]Cbl. The cells were homogenized in 10 mM Tris-HCl buffer consisting of 140 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride, and the homogenate was extracted with Triton X-100 (1%) for 12 h at 5 °C. Triton X-100 completely solubilizes TCII receptor (19).

Met Synthase Activity—A modification (20) of the method developed by Weissbach *et al.* (21) was used for measurement of Met synthase activity in cell extracts. This is the conventional assay and measures conversion of [^{14}C]CH$_3$-THF and Hcy to [^{14}C]Met.

The titanium citrate and NADPH assays are anaerobic assays. N_2 was bubbled through all reagent solutions, and the reaction was carried out in stoppered serum vials (air exchanged with N_2). Titanium citrate or NADPH was used as reductant, and the titanium citrate assay was run either in the presence (total) or absence (holo) of OHCbl, whereas Cbl was omitted from the NADPH assay. As in the conventional assay, [^{14}C]CH$_3$-THF was converted to [^{14}C]Met, and the radioactive Met was determined (22).

MCM Activity—The assay measures the conversion of methylmalonyl-CoA to succinyl-CoA. This assay was performed by an automated HPLC assay described by Riedel *et al.* (23). The sonicated cellular suspension in glycerol was transferred to a cooled rack in a Gilson ASPEC (Gilson Medical Electronics), which performed the enzyme reaction and sample injection. AdoCbl (total activity) or water (holoactivity) was added to the cell suspension, and the enzyme reaction was started by adding DL-methylmalonyl-CoA. After 5 min at 37 °C, the reaction was stopped by adding trichloroacetic acid, then the protein precipitate was removed by filtration, and the samples were injected on a reversed phase C18 column which separated the succinyl-CoA product from methylmalonyl-CoA.

CR Activity—Fractionation of cells was essentially performed according to the procedure described by Pezacka *et al.* (8). The washed cells (10 million cells/ml) in isolation buffer (10 mM Tris-acetate, 1.5 mM EDTA, 250 mM sucrose, 10 BAEE units/ml trypsin inhibitor, 90 nM aprotinin, 5 μM leupeptin, 1 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) were disintegrated in a Teflon-glass homogenizer. Whole cells, the nuclei, and cell walls were removed by centrifugation at 1500 × *g*. Mitochondria were pelleted by centrifugation of the supernatant at 10,000 × *g*, and the supernatant obtained was subjected to ultracentrifugation at 100,000 × *g* for isolation of microsomes. The mitochondria were kept in 150–200 μl of isolation buffer and frozen at −80 °C until analysis. Microsomes were resuspended in 150–200 μl of a 50 mM Tris buffer with 0.2 mM phenylmethylsulfonyl fluoride and kept on ice until analysis. The purity of the fractions was determined by the measurement of the following marker enzymes: NADH-cytochrome *c* reductase, glutamate dehydrogenase, lactate dehydrogenase, and fatty acyl-CoA dehydrogenase.

The CR activity (8) was determined at 40 °C by measuring the rate of conversion of OHCbl to Cbl(II) in the presence of NADH (0.2 mM). Absorbance was read at 526 nm, and the differential molar extinction coefficient of OHCbl to Cbl(II) was 5.6×10^3 M^{-1} cm^{-1}. Protein in the fractions was measured by the BCA assay using bicinchoninic acid (Pierce).

RESULTS AND DISCUSSION

Design—We investigated Cbl metabolism and function in two variants of a human glioma cell line, one is Met-dependent (P60) and the other is selected on the basis of growth in a Hcy medium and is Met-independent (P60H). The Met dependence of P60 cells developed during long-term culture in the presence of Met and folic acid, but such conversion only rarely occurs (data not shown). P60 cells did not attain Met-independent growth by culture in a medium containing Hcy (and no Met) and/or high folate, and repeated exposure to a demethylating agent was required to develop a Met-independent revertant (3). Also, change in the growth requirements of the P60H cells was never observed. Thus, both variants seem phenotypically stable.

The parameters investigated include key steps in a series of events from binding of Cbl at the cell surface (transport, level 1), cellular Cbl retention and metabolism (level 2), and finally the activities of the two Cbl-dependent enzymes, Met synthase and MCM (level 3) (Fig. 1). The functions were measured under standard culture conditions and under conditions assumed to
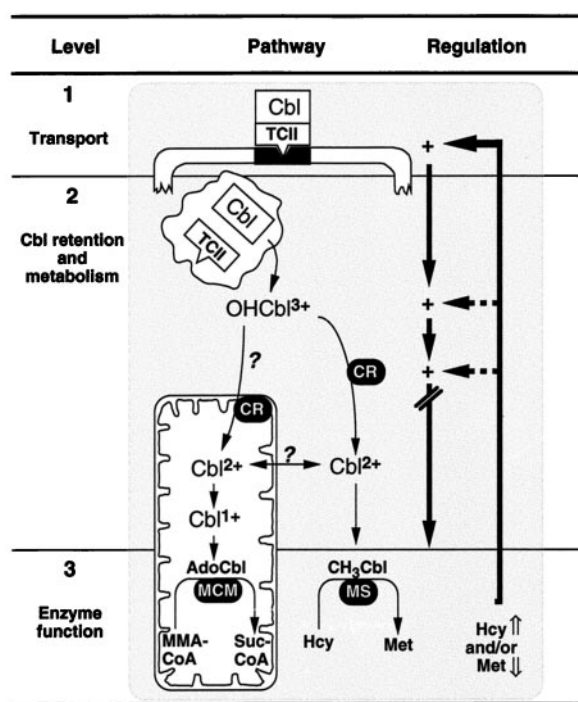
FIG. 1. **Cellular Cbl metabolism from uptake to enzyme function.** The possible sites affected by low Met/high Hcy are indicated, and the *break* represents the suspected site of defect of P60 cells in the proximity of CR. The *question marks* refer to the fact that there is uncertainty about the coordination between microsomal and mitochondrial CR.

pose a metabolic stress on the Met synthase pathway. Such stress was induced by transferring the cells to a Hcy medium which makes conversion of Hcy to Met essential for cell survival. We also exposed the cells to N_2O which irreversibly inactivates Met synthase (14).

TCII Receptor Activity (Level 1)—The TCII receptor expression level has been shown to respond to different physiological signals such as cell proliferation (24) and differentiation (25), intracellular Cbl status (25), and cortisone level (26). There are no data relating TCII receptor expression level to the catalytic activity of the two Cbl-dependent enzymes.

We determined the TCII receptor activity in P60H and P60 cells cultured in a Hcy medium and after N_2O exposure. The receptor activity was lower (22 fmol/10^6 cells) in P60 than in P60H cells (41 fmol/10^6 cell) when cultured under standard conditions (Met medium).

Exposure to N_2O by itself had no effect. However, transfer of the cells to a Hcy medium caused a marked increase in TCII receptor activity which was further increased by N_2O. This induction was particularly pronounced in the P60 cells, which responded to the combination of medium shift plus N_2O with a nearly 10-fold increase in activity, to 175 fmol/10^6 cells (Fig. 2).

Cobalamin Retention and Metabolism (Level 2)—In human melanoma cells, Liteplo *et al.* (4) demonstrated a relation between Met dependence and impaired Cbl retention despite normal uptake. In both P60 and P60H cell lines, we found a total Cbl level of about 500 fmol/10^6 cells, and CNCbl and OHCbl were the predominating Cbl species. However, CNCbl accounted for 20% of the total Cbl pool in the P60H cells and 37% in P60, suggesting a more extensive metabolism of CNCbl in P60H cells. The OHCbl content in P60H remained essentially unchanged in response to culture in Hcy medium and N_2O. In contrast, P60 cells transferred to Hcy medium showed

a dramatic increase in OHCbl content from about 250 to 600 fmol/10^6 cells (Fig. 2) and in total Cbl (data not shown). From these data, the following conclusions can be made. First, the high Cbl levels in P60 cells cultured in the Hcy medium are in agreement with the marked induction of the TCII receptor activity in these cells. Secondly, high OHCbl in P60H and P60 cells suggests that the rate of decyanation of CNCbl catalyzed by cyanocobalamin β-ligand transferase (8, 27) does not limit the provision of Cbl in either cell line. Finally, the accumulation of OHCbl in P60 cells suggest that the hypothetical block in Cbl traffic is located distal to the site of intracellular ligand transfer.

NADH-linked cobalamin reductase (CR) functions further down the metabolic route. It catalyzes the reduction of OHCbl(III) to Cbl(II) *in vitro* (8, 10) and is the last common step before Cbl is directed into two separate pathways, *i.e.* association with Met synthase (2, 9) or formation of AdoCbl which is the cofactor of MCM (11).

We measured NADH-linked CR activity in the microsomal and mitochondrial fractions of P60 and P60H cell lines. In a Met medium, both the microsomal and mitochondrial activities were lower in P60 compared with P60H cells. The activities were 16 ± 7 and 34 ± 17 in microsomes and 51 ± 28 and 139 ± 7 in mitochondria for P60 and P60H, respectively (values are average of three experiments $\pm$ S.D.). When cells were transferred from Met to a Hcy medium, the mitochondrial enzyme activities in the two cell lines were essentially unaffected whereas the activity of the microsomal enzyme was enhanced, especially in P60 cells. N_2O exposure was without effect (Fig. 2).

These data demonstrate for the first time specific induction of CR under conditions of metabolic strain of the Met synthase pathway. Selective response of microsomal CR serves to distinguish the microsomal CR from the mitochondrial isoenzyme. The up-regulation was most pronounced in the P60 cells and thus represents the third and presumably last process in a sequence of metabolic steps being more responsive in the P60 cells than in the P60H cells.

Enzyme Function (Level 3)—We have previously demonstrated in an intact cell assay that P60H but not P60 cells efficiently remethylate Hcy to Met when cultured in the Hcy medium (13). This can probably be related to the very low level of CH_3Cbl in the P60 cells, consistent with impaired function of Met synthase (3, 13).

Cells belonging to the *cbl*E complementation group are characterized by normal Met synthase activity when measured by a conventional assay, but reveal a functional Met synthase deficiency in an intact cell assay; hence, they resemble P60 cells. Recently, Gulati *et al.* provided evidence that *cbl*E cells have a defect in the auxiliary redox proteins that activate Met synthase. *cbl*E cells had normal activity as determined by the conventional assay or an assay using titanium citrate as a reductant, but low activity was measured when the physiological reductant, NADPH, was employed (28). In the present work, we investigated a possible Met synthase defect in the P60 cells by comparing the activity obtained by the three different assays (Table I). The titanium citrate assay gave the highest values, followed by the NADPH assay and the conventional assay. Independent of the assay, the enzyme activity in P60 relative to that in P60H cells was constant, and N_2O caused a substantial reduction (>60%) in both cell lines. Compared with the enzyme activities obtained by Gulati *et al.* (28), normal activity was detected in all assays including the NADPH assay in P60 cells, whereas P60H showed very high values. Notably, previous data have shown a frequent discrepancy between activity of Met synthase *in vitro* and remethylation of Hcy in
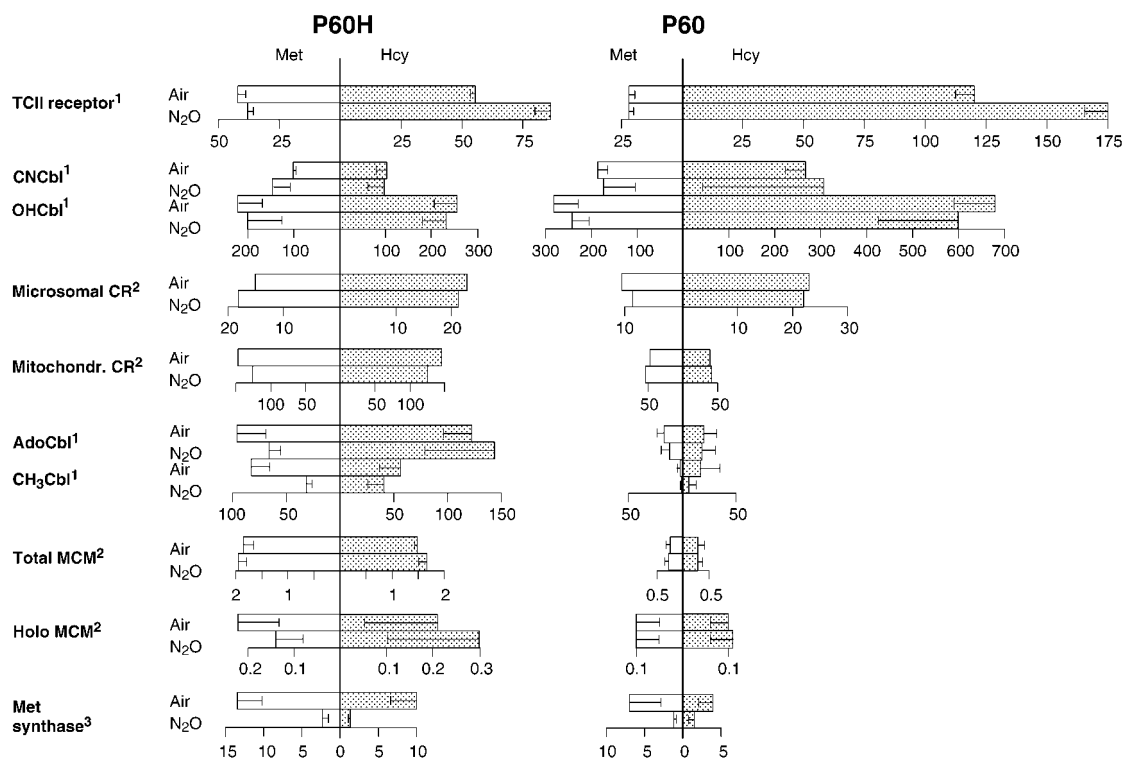
FIG. 2. **The functional and metabolic Cbl profile of the Met-independent P60H (*left panel*) and the Met-dependent P60 cells (*right panel*) cultured in either Met (*left, open bars*) or Hcy medium (*right, shaded bars*) and exposed to air or N₂O.** The profile is defined in terms of TCII receptor activity, cellular Cbls, microsomal and mitochondrial CR, holo and total MCM, and total Met synthase (conventional method) as indicated on the graph. The cells were grown for 48 h as described under "Materials and Methods." The data are mean of 3–4 experiments ± S.D. (Cbls, holo and total MCM, and Met synthase), 3–4 determinations (TCII receptor activity), or 2 determinations (CR). *1*, fmol/10⁶ cells; *2*, nmol/min/mg of protein; *3*, nmol/h/mg of protein.

TABLE I

Methionine synthase activity determined by different assays

After change of medium, the cells were preincubated for 3 h and then exposed to air or N₂O for 48 h. Cells were harvested for all three assays. In the conventional assay, dithioerythritol is used as reductant and antioxidant, whereas NADPH and titanium citrate are anaerobic assays. All assays measure the conversion to [¹⁴C]Met from [¹⁴C]CH₃THF and Hcy.

Methionine synthase assay	P60H cells		P60 cells	
	Air	N₂O	Air	N₂O
	pmol/min/mg protein			
Conventional	223 ± 53	38 ± 13	115 ± 68	18 ± 7
NADPH	415 ± 28	41 ± 6	113 ± 21	3 ± 2
Titanium citrate (total)	946 ± 51	259 ± 13	190 ± 21	83 ± 4
Titanium citrate (holo)	930 ± 11	196 ± 8	244 ± 8	31 ± 1

intact cells (1, 3, 13). Taken together, the activity profile obtained with different assay formats is not consistent with a mutant Met synthase or defective reductive activation in P60 cells.

It is well recognized that Cbl deficiency affects both total and holo MCM activity (29–31). Recently, Riedel *et al.* found that holo MCM in intact glioma cells is highly dependent on the level of its cofactor.[2] We found that the MCM activity, especially the total level, was markedly lower in P60 cells compared with the P60H cells (Fig. 2). Total Cbl in P60 cells (mainly CNCbl and OHCbl) was equal to (Met medium) or even higher than (Hcy medium) the level in P60H cells, whereas AdoCbl was low. This points to the level of AdoCbl rather than the level of total Cbl as the factor regulating MCM activity. Culture in Hcy or N₂O exposure had minimal effects on total and holo

MCM in the P60 cells, whereas the P60H cells responded to N₂O exposure with a slight decrease of holo MCM in a Met medium but an increase in a Hcy medium. The changes in holo MCM activity mirrored the alterations in AdoCbl level, further supporting the recent data from Riedel *et al.*[2]

Feedback Regulation of Cbl Transport—Our observations (Fig. 2) can be synthesized into a model for the regulation of Cbl metabolism in the glioma cell line (Fig. 1).

When the cells were grown in a Hcy medium, the TCII receptor activity increased. The response was further enhanced by N₂O and was particularly pronounced in the P60 cells which have low remethylation. This could be explained by feedback control at the level of TCII receptor expression mediated by the substrate and/or product of the Cbl-dependent Met synthase. The absence of an N₂O effect on TCII receptor activity in a Met medium (high cellular Hcy, high Met) and the high TCII expression in Hcy medium which is further enhanced by N₂O (high cellular Hcy, low Met) point to low Met as the regulatory signal. Enhanced Cbl transport may account for the high level of intracellular Cbl observed in P60 cells cultured in a Hcy medium, but the metabolic signal (low Met) may also directly affect Cbl metabolism and retention. Likewise, the enhanced activity of microsomal CR in P60 cells may be secondary to an increase in OHCbl content (substrate) or is caused by a direct positive control.

The Possible Site of the Metabolic Change in P60 Cells—The stimulatory effects of Hcy medium (especially when combined with N₂O) on the TCII receptor were most clearly demonstrated in Met-dependent P60 cells which also accumulated OHCbl during culture in a Hcy medium. The low level of both AdoCbl and CH₃Cbl, combined with consequent impaired function of both Cbl-dependent enzymes in the Met-dependent cells, provides the strongest evidence for an upstream location of the

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[2] B. Riedel, T. Fiskerstrand, H. Refsum, and P. M. Ueland, submitted for publication.

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defect, probably in proximity of the CR.

The alternative explanation of an isolated defect in CH_3Cbl synthesis combined with a cross-talk between the two Cbl-dependent enzyme systems is not in agreement with data on Cbl mutant cells. *cbl*A and -B are mutants that affect MCM, but the CH_3Cbl content and the activity of Met synthase are normal. *cbl*G and -E mutants have a low Met synthase activity and cellular level of CH_3Cbl, but the AdoCbl content is normal (5). Although some cases with *cbl*G and -E disease have slightly decreased activity of MCM in intact cells, the levels are still much higher than those observed in *cbl*A and -B, and patients with *cbl*G and -E disease have no symptoms indicating dysfunction of MCM (32).

The cause of the metabolic difference in the P60 cells has not been identified. Notably, the low mitochondrial CR activity in P60 cells relative to P60H cells (Fig. 2) was equal to the reduction observed in *cbl*C cells relative to control fibroblasts (10). The authors suggested that the low CR activity in the *cbl*C cell line was not due to reduction in levels of the enzyme but in its affinity for NADH cofactor. This enzyme has still not been cloned, but our findings suggest that gene(s) coding for CR are candidates for mutation screening in Met-dependent cancer cells.

CONCLUSION

In conclusion, the present work provides evidence for a regulatory network involving Cbl transport, uptake, metabolism, and Met synthase function. A positive feedback control of TCII receptor expression by Met depletion may represent a key component of this system. The Met dependence of the P60 cells seems to be related to disruption of this network in the proximity of CR. It is not clear whether the reductive steps required for AdoCbl and CH_3Cbl formation are carried out by the same reductase system, and further elucidation of the molecular defect responsible for the Met dependence will depend on additional knowledge of the enzymes involved in intracellular Cbl reduction.

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