3-Hydroxy-3-methylglutaryl coenzyme A lyase: targeting and processing in peroxisomes and mitochondria

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Abstract 3-Hydroxy-3-methylglutaryl coenzyme A lyase (HL, E.C. 4.1.3.4) has a unique dual localization in both mitochondria and peroxisomes. Mitochondrial HL (~31.0 kDa) catalyzes the last step of ketogenesis; the function of peroxisomal HL (~33.5 kDa) is unknown. On density gradient fractionation, normal human lymphoblasts contain both peroxisomal and mitochondrial HL whereas in lymphoblasts from a patient with Zellweger syndrome, in which functional peroxisomes are absent, only the mitochondrial HL isoform was present. To study the kinetics of the dual targeting of HL, we performed pulse-chase experiments in normal and Zellweger cells. Pulse-chase studies revealed a biphasic curve for processing of the HL precursor. The first phase, with a calculated half-life of ~3 h in both normal and Zellweger fibroblasts and lymphoblasts and in HepG2 cells, presumably reflects mitochondrial import and processing of the precursor; the second ($t_{1/2}$, 12–19 h) is present only in normal cells and presumably represents the half-life of peroxisomal HL. The half-life of mature mitochondrial HL was 14 to 19 h in both normal and Zellweger cells. Studies of the HMG-CoA lyase precursor in isolated rat mitochondria showed a rate of processing ~2.6-fold lower than that of the ornithine transcarbamylase precursor.—Ashmarina, L. I., A. V. Pshezhetsky, S. S. Branda, G. Isaya, and G. A. Mitchell. **3-Hydroxy-3-methylglutaryl coenzyme A.** ***J. Lipid Res.*** **1999.** 40: **70–75.**

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In the mitochondrial matrix, 3-hydroxy-3-methylglutaryl coenzyme A lyase (HL, EC 4.1.3.4) catalyzes the last step of lipid energy metabolism, the cleavage of HMG-CoA to acetyl-CoA and the ketone body, acetoacetate. In human and mouse liver, we have shown HL to be present in peroxisomes as well as in mitochondria (1). The role of peroxisomal HL is unknown but is notable in view of the increasing evidence of the role of peroxisomes in cholesterol synthesis from HMG-CoA (2). In humans, the autosomal recessive deficiency of HL can cause episodes of hypoglycemia and metabolic acidosis (3). The primary structure of the HL precursors of human and mouse, deduced from cDNA and gene sequences (4, 5) contains both a potential mitochondrial targeting sequence at the N-terminus and a potential peroxisomal targeting tripeptide, Cys-Lys-Leu, at the C-terminus. In humans and mice, peroxisomal HL differs from the mitochondrial isoform in having a molecular mass ~2.5 kDa greater and a more basic pI value. We have hypothesized that peroxisomal HL is identical to the mitochondrial HL precursor, retaining the N-terminal targeting sequence (6). Recombinant HL precursor is similar to mature mitochondrial HL in its enzymatic properties, but exists as a monomer, in contrast with mitochondria HL, which is a dimer (6).

In order to understand the mechanism of the dual targeting of HL to mitochondria and peroxisomes, we have compared normal cells with cells from patients with Zellweger syndrome. Zellweger syndrome is an autosomal recessive disorder resulting from deficiency of peroxisomal biogenesis (7).

MATERIALS AND METHODS

Cells and cell culture

Three cell types were studied: fibroblasts from normal controls and from a patient affected with Zellweger syndrome (NIGMS Human Genetic Mutant Cell Repository line GM00228A, complementation group 1), lymphoblasts from normal controls and from a Zellweger syndrome patient of complementation group 4 (a gift from Anne Moser), and human liver carcinoma (HepG2) cells. Fibroblasts and HepG2 cells were cultured in Eagle's minimal essential medium (EMEM, Mediatech), and lymphoblasts were cultured in RPMI 1640 medium (RPMI, BRL Life Technolo-

Abbreviations: HBSS, Hank's balanced salt solution; HL, HMG-CoA lyase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; mHL, mature HL; pHL, precursor HL; NP40, nonidet P-40; OTC, ornithine transcarbamylase; pOTC, precursor of OTC; PMSF, α-toluensulfonyl fluoride.

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gies Inc). In each case, the medium was supplemented with 20% fetal calf serum and antibiotics.

Fractionation of lymphoblasts

Human lymphoblasts (~10^8 cells) were centrifuged at 800 *g* for 10 min at room temperature and washed twice with PBS (phosphate-buffered saline: 0.15 M NaCl, 2.7 mM KCl, 3 mM Na_2HPO_4–KH_2PO_4, pH 7.4). The cells were suspended in 3 ml of ice-cold homogenization solution (0.25 M sucrose, 1 mM Tris-HCl, pH 7.5, 1 mM EDTA) and protease inhibitors (leupeptin, antipain and pepstatin) were added to a final concentration of 1 μg/ml of each. The lymphoblasts were homogenized in a 5-ml Teflon-glass Potter-Elvehjem homogenizer followed by centrifugation at 1500 *g* for 10 min. The supernatant was subjected to equilibrium density centrifugation on a linear 24.2–54.5% (w/w) sucrose gradient using a SW-41 rotor (Beckman) at 28,000 rpm and 4°C for 150 min. After centrifugation the gradient was divided into 15 fractions, the least dense being designated fraction 1.

Immunoblotting

Western blotting was performed as described using a rabbit anti-recombinant human HL serum (1).

Metabolic labeling

Human fibroblasts and HepG2 cells, grown to confluency in 75-cm^2 culture flasks (~10^6 cells), were washed twice with Hank's balanced salt solution (HBSS), then incubated for 2 h in methionine-free Dulbecco's Modified Eagle's Medium (D-MEM, Gibco BRL) supplemented with l-glutamate and sodium pyruvate, and finally for 40 min in 5 ml of the same medium supplemented with [^{35}S]methionine (DuPont), 0.1 mCi/ml. The radioactive medium was then removed, and the cells were washed twice with HBSS and chased at 37°C in EMEM supplemented with 20% fetal calf serum.

Human lymphoblasts (~10^6 cells) were washed twice with HBSS, placed for 2 h in methionine-free RPMI 1640 medium (Gibco BRL), labeled for 40 min with [^{35}S]methionine, (0.1 mCi/ml), washed twice with HBSS, and chased at 37°C in RPMI 1640 medium supplemented with 20% fetal calf serum.

At the time indicated in the figure legends, the cells were placed on ice, washed twice with ice-cold PBS, then lysed for 30 min on ice in 1 ml of 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.5% nonidet P-40 (NP-40); 5 μg/ml leupeptin, and 0.1 mM α-toluensulfonyl fluoride (PMSF). The lysate was collected and centrifuged at 13,000 *g* for 10 min to remove debris. For some experiments indicated in the text, cells were separated into supernatant and particulate fraction by digitonin permeabilization as described by Mori and co-workers (8).

Immunoprecipitation, electrophoresis and quantitation of mHL and pHL

One ml of whole cell lysate was incubated for 4 h with preimmune serum at a final dilution of 1/20. Then the pellet obtained from 300 μl of Pansorbin Cells (Calbiochem) was added and the resulting suspension was incubated for 2 h at 4°C, followed by centrifugation for 10 min at 13,000 *g*. Supernatants were incubated overnight with the previously described (1) rabbit anti-recombinant human HL polyclonal antiserum in a 1/100 final dilution, then incubated for 2 h at 4°C with the pellet from 100 μl of Pansorbin Cells and precipitated as above. The pellet was washed three times with 1 ml of a solution containing 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS and 0.1% PMSF. The antigens were eluted from the pellet by the addition of 100 μl of a buffer containing 0.1 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 M DTT, and 0.02% (w/v) bromphenol blue. The peptides were denatured by boiling for 5 min and subjected to SDS-PAGE (9). The following ^{14}C-labeled protein molecular mass markers (Amersham) were used: lysozyme (14.3 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa), and phosphorylase b (97 kDa). The gels were fixed in acetic acid–isopropanol–water 10:50:40, soaked for 30 min in Amplify™ solution (Amersham), vacuum dried at 60°C, and analyzed either by autoradiography and scanning densitometry or by quantitative fluorometry on a PhosphorImager SI analysis screen (Molecular Dynamics) using software supplied by the manufacturer. To calculate the amounts of pHL and mHL, the measured intensities of the bands were corrected for the number of methionine residues present in each molecule (pHL, 11 methionine residues; mHL, 8).

Calculation of half-lives for pHL and mHL

The half-lives of pHL and mHL were calculated from the slopes of regression lines in semilogarithmic plots (e.g., Fig. 4).

Enzyme assays

HL was assayed spectrophotometrically using HMG-CoA as a substrate (10). The fractions obtained by density centrifugation or from the digitonin permeabilization experiment were assayed for the activities of the following marker enzymes: catalase (11) (peroxisomes); glutamate dehydrogenase (12) (mitochondria), and phosphoglucose isomerase (13) (cytosol). In each assay, one unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol of substrate/min, and relative activity is the percentage of total cellular activity found in the subcellular fraction. Proteins were assayed according to Bradford (14) with bovine serum albumin (Sigma) as a standard.

Mitochondrial import assay

To synthesize HL RNA for in vitro translation, we used the HLH1 plasmid, which contains a full-length human HL cDNA and a 5-prime flanking T7 RNA polymerase site (4). Mitochondrial isolation and import reactions were performed essentially as described (15). Specifically, 4 μl of freshly isolated rat liver mitochondria (20 mg/ml) was mixed with 6 μl of radiolabeled in vitro synthesized HL precursor peptide (pHL1) and incubated at 27°C. Aliquots were withdrawn from the import reaction at 0, 2, 10, and 20 min. In a control experiment after 20 min, the reaction was treated with trypsin (400 μg/ml) for 5 min at 4°C. Soybean trypsin inhibitor (1 mg/ml final) was then added and the reaction mixture was incubated for 5 min at 4°C. The mitochondrial pellet and postmitochondrial supernatant were separated by centrifugation at 12,000 *g* for 5 min at 4°C. The human OTC precursor was used as a positive control in parallel import reactions. pHL and pOTC import reactions were then analyzed by SDS/PAGE and autoradiography and quantitated by densitometry.

RESULTS AND DISCUSSION

Figure 1 shows the distribution of HL and marker enzyme activities in subcellular fractions from normal (Fig. 1a) and Zellweger (Fig. 1b) lymphoblasts. As expected (16), in Zellweger lymphoblasts, the peroxisomal marker, catalase, is cytoplasmic whereas the pattern of activity of the mitochondrial marker, glutamate dehydrogenase, is similar to that of normal cells. Normal lymphoblasts have two distinct peaks of HL activity that coincide with the activity peaks of the mitochondrial and peroxisomal marker enzymes. We observed a similar bimodal distribution of HL activity in subcellular fractions of mouse and human liver (1). In contrast, in Zellweger lymphoblasts (Fig. 1b) HL activity is restricted to a single peak that coincides with

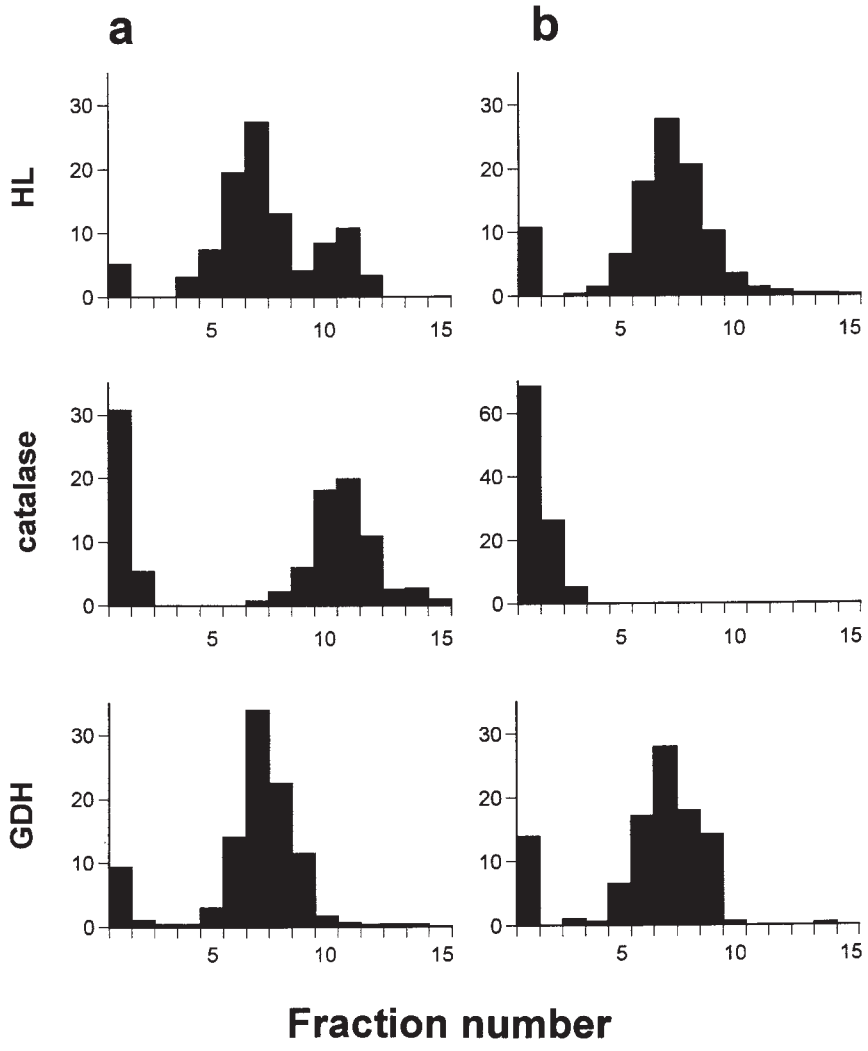

Fig. 1. Distribution of enzyme activities in equilibrium density centrifugation of (a) normal control and (b) Zellweger lymphoblasts. The X-axis represents sucrose gradient fractions, fraction 1 being the least dense. The Y-axis represents the relative activity of the enzymes indicated in each panel. Abbreviation: GDH, glutamate dehydrogenase.

the mitochondrial marker. As in mouse and human liver (1) the estimated molecular mass of the HL immunoreactive band in the peroxisomal fraction (~33.5 kDa, **Fig. 2**, lane 2), is 2.5 kDa greater than that in mitochondrial fraction (~31.0 kDa, Fig. 2, lane 1), and close to the calculated 2.8 kDa difference between the deduced molecular masses of precursor HL (34.4 kDa) and mature HL (31.6 kDa). In Zellweger cells, only the 31.0 kDa HL isoform was present in the mitochondrial fraction (Fig. 2, lane 3).

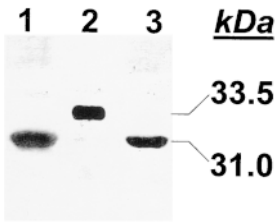

Fig. 2. Western blot of mitochondrial and peroxisomal peak fractions from equilibrium density centrifugation of human lymphoblasts: lane 1, mitochondrial fraction, normal lymphoblasts (50 μg of protein); lane 2, peroxisomal, normal (50 μg); lane 3, mitochondrial, Zellweger (20 μg).

To further test the processing of mitochondrial and peroxisomal forms of HL we performed metabolic labeling studies in human lymphoblasts, fibroblasts, and HepG2 cells. **Figure 3** shows an example of a 22-h pulse-chase experiment with normal and Zellweger fibroblasts. Two fragments (M_r ~33.8 and ~31.3) were precipitated by anti-HL antibodies. As in Fig. 2, the observed masses coincide with those of precursor and mature HL; the fragment of ~29kDa, which is also detectable at 14 h and 22 h for normal fibroblasts, and at 4 h for Zellweger fibroblasts may represent a product of HL degradation. Initially there is a preponderance of pHL, but within 4 h this is reversed. In this and other studies, the ratio between pHL and mHL appeared to be constant after approximately 14

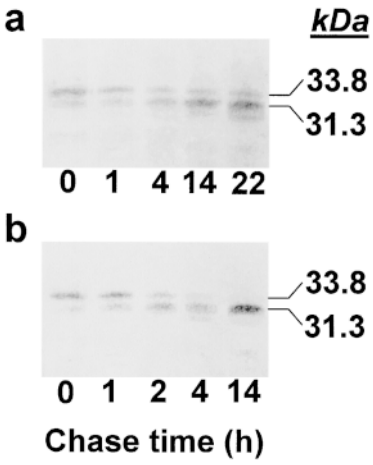

Fig. 3. Pulse-chase studies of HL in normal (a) and Zellweger (b) fibroblasts. The chase times (h) and M_r estimates for pHL (33.8) and mHL (31.3) are shown.

h. In contrast, in pulse-chase studies of Zellweger fibroblasts (Fig. 3b), the initially intense 33.8 kDa pHL fragment was undetectable by 14 h of chase.

In **Fig. 4**, these results are shown graphically for all cell types studied. In normal fibroblasts, lymphoblasts, and HepG2 cells, pHL decreases to 16–20% of total HL by ~25 h and this proportion does not change thereafter. In Zellweger lymphoblasts and fibroblasts, pHL is nearly undectable by 20 h of chase. The estimate that 16–20% of cellular HL peptide is present as pHL in normal fibroblasts, lymphoblasts, and HepG2 cells is of the same order as our previous calculation that ~6% of mouse and human liver HL activity is peroxisomal (1).

Observation of a stable high molecular weight HL species in normal cells could be explained by retention of

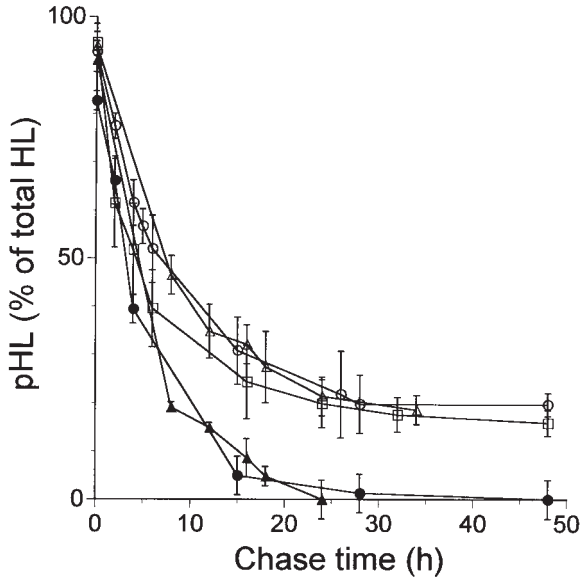

Fig. 4. pHL processing in HepG2 cells (□), normal (○), and Zellweger (●) fibroblasts, and normal (△) and Zellweger (▲) lymphoblasts. Each point represents an average of at least 3 values ± standard error of the mean.

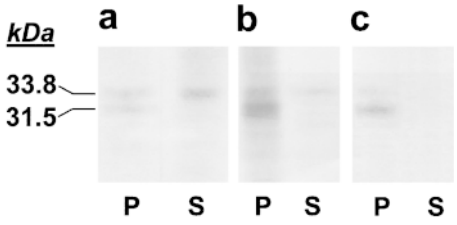

Fig. 5. Distribution of pHL and mHL in particulate and soluble fractions from fibroblasts. After a 40-min pulse, digitonin permeabilization was performed either (a) immediately, or (b) after 3 h, or (c) after 22 h chase period. P, particulate fraction; S, soluble fraction.

pHL in the cytoplasm, slow uptake by mitochondria, and/or long half life in peroxisomes. To test whether pHL is in the soluble fraction for prolonged periods, we performed digitonin permealization and subcellular fractionation of the [^{35}S]methionine-labeled fibroblasts and also determined marker enzyme activities in similarly treated unlabeled fibroblasts. The conditions used to disrupt the cell membrane are known to leave organelles intact (8). We confirmed this in a control experiment, showing that 89% of the activity of the cytosolic marker, phosphoglucose isomerase, is recovered in the soluble fraction, in contrast to ~5% of HL activity and ~8% of the activity of the peroxisomal marker, catalase. As shown in **Fig. 5**, pHL is initially present in both soluble and particulate fractions (Fig. 5a). At 3 h of chase (Fig. 5b) it is less intense, but still detectable in the soluble fraction and at 22 h (Fig. 5c) all pHL is particulate.

We performed kinetic modeling of pHL processing and degradation in human fibroblasts (**Fig. 6a**). The kinetic curve for pHL in normal fibroblasts is biphasic, with an initial rapid decrease ($t_{1/2}$~3 h) followed by a slower decline ($t_{1/2}$~19 h). The same plot for Zellweger fibroblasts shows a rapid monophasic decline ($t_{1/2}$~3.3 h), similar to the first phase observed in normal fibroblasts. For mHL, the results in normal and Zellweger fibroblasts are superimposable (Fig. 6b), showing an initial rapid increase, then a slow decline with a half-life of ~16 h.

We observed a similar pattern of pHL and mHL processing in HepG2 cells and in lymphoblasts, although in HepG2 cells the half-lives of pHL (~12 h) and of mHL (~13.5 h) were shorter than those of fibroblasts. The maturation of pHL ($t_{1/2}$~3 h) appears to be much slower than that of other mitochondrial precursors (~30 sec to 10 min) (17–20). Furthermore the half-life for peroxisomal uptake of precursors has been estimated to range from 4 to 15 min (21) showing that the uptake of pHL is slower than that for most peroxisomal proteins.

To directly test mitochondrial import and processing, we incubated in vitro synthesized HL precursor, pHL1, with isolated mitochondria. The results showed that pHL1 can be imported and processed by mitochondria (**Fig. 7**). In a control experiment using trypsin treatment, we showed that the processed form of pHL1 (i.e., mHL1) was associated with the mitochondrial pellet and was protected from trypsin digestion, while pHL1 remaining in

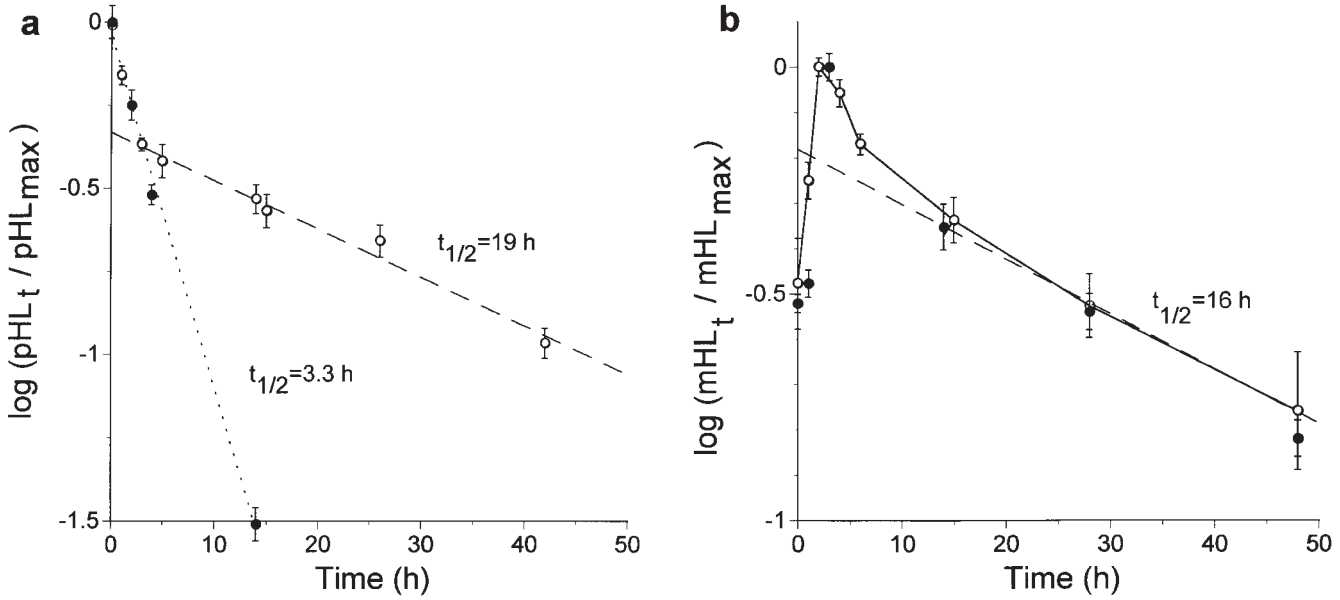

Fig. 6. Disappearance of fibroblast pHL and mHL during pulse-chase experiments: (a) pHL; (b) mHL. Both panels show semilogarithmic plots of the relative amount of HL species remaining in the fibroblasts versus chase time. The calculated half lives are shown. Plots represent regression lines with *r* values as follows. Normal cells: mHL, −0.98; pHL, −0.993. Zellweger cells: mHL, −0.998; pHL, −0.998. Normal fibroblasts are plotted as open circles (○); Zellweger fibroblasts, as filled circles (●).

the supernatant was digested to two smaller products (T in Fig. 7). The difference in M_r (2.3 kDa) observed in vitro between pHL1 and mHL1 was similar to that observed in the above pulse-chase studies and in human liver (1). Similar experiments were performed with the in vitro synthesized precursor of a control mitochondrial enzyme, human ornithine transcarbamylase (pOTC, Fig. 6b). For pHL1, the calculated rate of processing was 2.6-fold lower than that of pOTC (**Table 1**).

We considered whether the slow maturation of pHL might be caused by interference by peroxisomes. Of note, membranous "peroxisomal ghosts" are well documented in Zellweger cells (22, 23), and can contain peroxisomal proteins such as the 70-, 26-, and 22-kDa integral membrane proteins and 3-ketoacyl-CoA thiolase (24, 25). However, peroxisomal matrix proteins, which like HL have a C-terminal targeting motif, have not been shown to associate with these structures. Specifically, no uptake of proteins with the C-terminal targeting sequence was observed in ghosts from patients of complementation groups 1 and 4, which we used in our experiments (26, 27). Therefore, it is unlikely that peroxisomal interference causes the slow cleavage of pHL.

We therefore examined the sequence of the mitochondrial leader of pHL. The uptake and processing of mitochondrial precursors is sequence dependent, being enhanced by the presence of amphiphilic alpha helices containing positively charged amino acid residues, and being greatly influenced by the tertiary structure of the remainder of the peptide (28). Although the 27-residue HL mitochondrial targeting peptide contains five dibasic amino acids and no acidic residues, the prediction of its secondary structure using the Prot Scale and nnPREDICT packages of the Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva) revealed neither a strong tendency to alpha helix formation nor marked amphiphilicity. Furthermore, the N-terminus of pHL was not recognized as a mitochondrial targeting sequence by the PSORT package

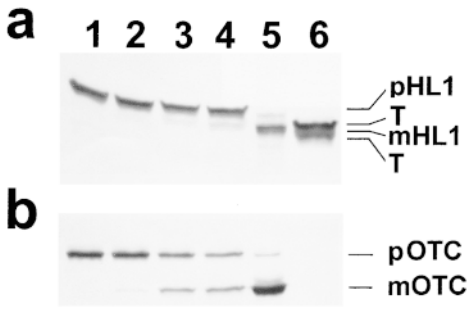

Fig. 7. Mitochondrial import studies of (a) pHL1 and (b) pOTC. The lanes correspond to the following incubation times: (1) zero time; (2) 2 min; (3) 10 min; (4 to 6) 20 min. In lanes 5 and 6, trypsin digestion was performed after the 20-min incubation and the sample was divided into pellet (lane 5) and supernatant (lane 6) fractions. Positions of precursors (pHL1, pOTC), processed enzymes (mHL1, mOTC) as well as of products of trypsin digestion (T) are shown.

TABLE 1. In vitro processing of pHL1 and pOTC

	Time			
	0 min	2 min	10 min	20 min
		%		
pHL1	0	3.3	10.6	19.2
pOTC	0	7.2	39.6	50.1

The values indicate the signal intensity of the processed peptide, expressed as a percentage with respect to the combined signal intensity of the precursor and processed peptides.

for prediction of protein sorting signals. To date, we have no evidence that the N-terminus of pHL provides a strong mitochondrial targeting signal, and it will be of interest to study this directly. The lengthy cytoplasmic retention of pHL may be due to its presence in the free form or in a form bound to cytoplasmic protein. One hypothesis that could be addressed specifically is that pHL associates in a prolonged fashion with the docking protein for the peroxisomal targeting sequence receptor (29).

We speculate that the dual localization of HL within cells results from an intricate balance between mitochondrial and peroxisomal uptake. A precedent exists in the case of alanine:glyoxylate aminotransferase, which is found variously in peroxisomes and mitochondria in different mammalian species (30). A major cause of alanine: glyoxylate aminotransferase deficiency in humans is a mutation that reduces peroxisomal uptake, diverting the enzyme from its normal peroxisomal location to mitochondria. Interestingly, in the case of HL, a small group of patients present with symptoms suggestive of an organic acidemia and have elevated urinary levels of 3-hydroxy-3-methylglutaric acid, but normal values of HL activity (31). Distribution of HL exclusively to peroxisomes is one explanation for this finding, and could be studied by the methods described here. Conversely, if peroxisomal HL proves to have a biological function, the mistargeting of HL exclusively to mitochondria might also cause a disease phenotype.

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