Human cystathionine γ-lyase: developmental and *in vitro* expression of two isoforms

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Cystathionine γ-lyase (CGL) is the last enzyme of the trans-sulphuration pathway, which converts methionine into cysteine. To study the possible differences in enzymic activity of the two human cystathionine γ-lyase isoforms characterized earlier, these were separately expressed in human kidney embryonic 293T cells. Furthermore, developmental changes in the expression of the two mRNA forms as well as the enzymic activity in human liver were studied, as it has been postulated that a change in the relative expression of CGL isoforms causes the postnatal increase in CGL activity. Transfection with the longer isoform increased the CGL activity 1.5-fold, while the activity of the cells transfected with the shorter form did not differ from the basal activity. In human liver samples, CGL activity was only detected in adult tissue (68 ± 9 nmol of cysteine/h per mg of protein), whereas activity in fetal, premature and full-term neonatal liver tissue was undetectable. In contrast, strong mRNA expression of both mRNA isoforms was detected from the 19th gestational week onwards and the longer form of CGL appeared to be predominant. The expression of the two mRNA forms varied in parallel. In conclusion, we have shown that only cells overexpressing the longer form of CGL have increased activity, and CGL appears to be regulated at the post-transcriptional level during development.

Key words: alternative splicing, cysteine, fetal development, liver, pyridoxal 5′-phosphate.

INTRODUCTION

Mammalian cells are capable of synthesizing L-cysteine from L-methionine via the trans-sulphuration pathway [1]. The last enzyme of this pathway, cystathionine γ-lyase (CGL, γ-cystathionase, EC 4.4.1.1), catalyses the conversion of L-cystathionine into L-cysteine, α-ketobutyrate and ammonia. The trans-sulphuration pathway is essential for an adequate supply of cysteine in rat hepatocytes *in vitro* [2,3]. In addition to being an amino acid component of polypeptide chains, cysteine is needed for synthesis of GSH, a major intracellular antioxidant [4].

It has been shown that CGL activity [5], as well as immunoreactive protein [6], is absent from human fetal liver. In rat liver, the activity is low during fetal development but increases rapidly during the last 3 days of gestation [7]. The rate of GSH synthesis from methionine is six times lower in fetal than in adult rat hepatocytes, and it has been concluded that the difference was due to the low CGL activity [8]. In premature infants, plasma cysteine levels are much lower than in full-term newborns, also suggesting low CGL activity [9].

Both human [10] and rat [11] cDNAs for CGL have been cloned. In human, two forms of mRNA for CGL have been characterized, of which the shorter form has an internal deletion of 132 bp. It is not known whether these two forms are products of different genes or splice variants. It was proposed that the subunit composition of the tetrameric CGL regulates the activity, and that the developmental changes in CGL activity are caused by a change in relative expression of the two isoforms [10].

In the present study, we have expressed CGL isoforms in a eukaryotic expression system to assess the possible differences in enzymic activity. Furthermore, we have studied changes in the expression of the two forms in human liver during development. We also present evidence that the isoforms are splice variants.

EXPERIMENTAL

Recombinant plasmids

Human CGL cDNA (HCL-1, GenBank accession number 52028) was obtained from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.; catalogue number 79761). This cDNA clone represents the shorter CGL isoform. However, the original clone obtained from ATCC lacked the 3′ end (nt 1106–1194) of the reported sequence of the short form of CGL, CGL-S [10]. The missing nucleotides of the coding sequence, a Kozak consensus translation-initiation sequence and restriction sites were added to the sequence by PCR. The sequences of the primers used were as follows: forward, 5′-CTCTGGTACCGCG-ACC*ATGCAGGAAAAAGACGCCTCCTC*-3′ and reverse, 5′-CGTCGGTACC*CTAGCTGTGAATTCTTCCACTTGGAGG-GTGTGC*-3′ (sequences corresponding to human CGL are italicized and *Kpn*I restriction sites are underlined). pcDNA-CGL-S was created by cloning the resulting DNA fragment into the *Kpn*I site of mammalian expression vector pcDNA3 (Invitrogen).

In order to clone the longer isoform of CGL, CGL-L, 1 μg of total human liver RNA was reverse transcribed using random hexamer primers (Promega) and PCR amplified as above. The CGL-S and CGL-L clones were sequenced using an ABI prism automated sequencer (Perkin-Elmer).

Cell culture and transfections

293T cells (human embryonic adenovirus-transformed kidney cells, expressing simian virus 40 large T antigen) were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were seeded at a density of 4×10^6/10-cm plate 20 h before transfection and grown at 37 °C in a humidified atmosphere with 5% CO_2. Transient

Abbreviations used: CGL, cystathionine γ-lyase; CGL-S, cystathionine γ-lyase, short form; CGL-L, cystathionine γ-lyase, long form; CGS, cystathionine γ-synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PLP, pyridoxal 5′-phosphate; RT, reverse transcriptase.

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transfections were performed using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Cells were transfected with 6 μg of pcDNA-CGL-S, pcDNA-CGL-L or pcDNA3. Transfection efficiency was monitored by co-transfection with 1.5 μg of pCMVβ β-galactosidase expression vector (Clontech) and measurement of β-galactosidase activity as described in [12]. Cells were harvested by scraping 48 h after transfection, washed with PBS and pelleted by centrifugation. The pellets were either used immediately for further analyses or snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

CGL activity

CGL activity was measured as described previously [7]. For activity measurements, the cells were suspended in ice-cold 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. After three freeze-thaw cycles the non-soluble debris was spun down and the supernatant used for activity measurement. The frozen liver samples were homogenized in 30 mM sodium phosphate buffer (pH 7.0), centrifuged at 14000 ***g*** for 30 min at 4 °C, and filtered through P-10 gel filtration columns (Amersham Pharmacia Biotech) to remove endogenous amino acids. The specificity of the assay was confirmed using propargylglycine, an inhibitor of CGL.

Northern blotting

Total RNA was extracted from the 293T cells using RNeasy mini kit (Qiagen) and fractionated on agarose/formaldehyde gels at 10 μg/lane. Following capillary transfer on to nylon filters (Hybond-N, Amersham Pharmacia Biotech), the blots were hybridized using standard methods [13] with ^{32}P-labelled (DuPont) complementary RNA probe corresponding to nt 874–1106 of the published sequence [10]. Following hybridization and washes, the filters were exposed to Kodak BioMax MR autoradiography films (Eastman Kodak Co.). The filters were stripped and reprobed with ^{32}P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA probe transcribed from the p-TRI-GAPDH plasmid (Ambion).

RNA isolation from human liver, RNAse protection and reverse transcriptase (RT)-PCR

Fetal tissue samples were obtained from legal abortions. Neonatal liver samples (gestational age 26–42 weeks) were from autopsies performed within 12 h of death. Adult liver tissue was obtained from partial liver transplantations. The procedures for obtaining human samples were approved by the Ethical Committee of The Hospital for Children and Adolescents (Helsinki, Finland).

Total liver RNA was extracted by the guanidinium thiocyanate/caesium chloride method [14] and quantified using the ribonuclease-protection assay (RPA II, Ambion). To generate templates for single-stranded radiolabelled probes, 301-bp and 169-bp fragments of CGL-L and CGL-S were amplified using the forward primer 5′-GCAAGTGGCATCTGAATTTG-3′ and the reverse primer 5′-CCCATTACAACATCACTGTGG-3′ flanking the deletion site. The resulting fragments were cloned into the pCR 2.1 cloning vector (Invitrogen) and digested with *Spe*I. Using T7 RNA polymerase and [^{32}P]UTP, these vectors yielded 397- and 265-bp radiolabelled run-off transcripts and 299- and 167-bp protected fragments for CGL-L and CGL-S, respectively. To normalize for RNA content, human β-actin was used as a standard. pTRI-β-actin (Ambion) digested with *Dne*I and transcribed with SP6 RNA polymerase yields a 129-bp protected fragment. Following hybridization with total cellular RNA and incubation with RNAse, fragments were separated on a 5% polyacrylamide/8 M urea gel and exposed to autoradiography film. The X-ray films were scanned (Microtek ScanMaker) and analysed with the NIH-Image 1.61 analysis software (National Institutes of Health, Bethesda, MD, U.S.A.).

For RT-PCR, liver RNA samples were reverse transcribed and amplified using the same primers that were used for creating probes for the ribonuclease-protection assay.

PCR of genomic DNA

Our assumptions were that the two CGL forms are splice variants and that the 132-bp fragment missing from CGL-S is flanked by two introns. DynaZyme EXT DNA Polymerase (Finnzymes) was used to obtain the PCR fragments. For the amplification of the 5′ intron, 100 ng of human genomic DNA was amplified using forward primer 5′-GCAAGTGGCATCTGAATTTG-3′ and reverse primer 5′-ACAATATGTGCACAGCCTTC-3′. The resulting $\approx$ 2.3-kb fragment was cloned into the pCR-XL-TOPO vector (Invitrogen) and sequenced from both ends of the insert. The 3′ intron was amplified using forward primer 5′-AACCCCACCCAGAAGGTGATTG-3′ and reverse primer 5′-GCAGAATACATAGAAATATCAGCTCC-3′. PCR amplification resulted in an $\approx$ 5-kb DNA fragment, which was then cloned and sequenced as above.

Statistical analysis

Data are expressed as means $\pm$ S.E.M., and groups were compared using Student's *t* test. Linear regression was used to assess the correlation between the mRNA expression of CGL-S and CGL-L. Statistical analyses were performed with the SPSS 8.0 program (SPSS, Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

Cloning and transient expression of human CGL isoforms

After 30 cycles of PCR amplification, two bands (1245 and 1113 bp) were visible on ethidium bromide-stained agarose gels (Figure 1). Of these, the 1245-bp product was excised from the gel, restriction-digested with *Kpn*I and cloned into a pcDNA3 vector. The sequence of the cDNA was identical to the reported sequence

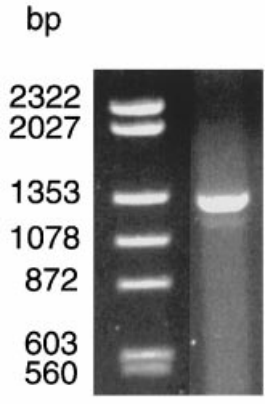

Figure 1 Amplified RT products from human liver total RNA

The amplified RT products were separated on a 1% agarose gel. Lane 1, λ-*Hin*dIII digest of λDNA and *Hae*III digest of ϕX174 DNA used as molecular-mass markers. Lane 2, ethidium bromide-stained products.

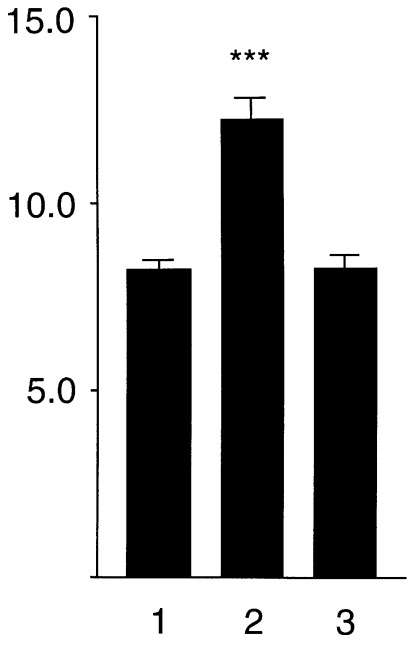

Figure 2 Total CGL activity from 293T cells transfected with the two CGL isoforms

Lane 1, control cells were transfected with 6 μg of an empty pcDNA3 vector; lane 2, cells transfected with 6 μg of pcDNA-GCL-L; lane 3, cells transfected with 6 μg of pcDNA-GCL-S; $n = 11$ in each group. Samples are from four independent experiments. Means ± S.E.M. are given. ***, Indicates a significance difference from control cells at the level of $P < 0.001$.

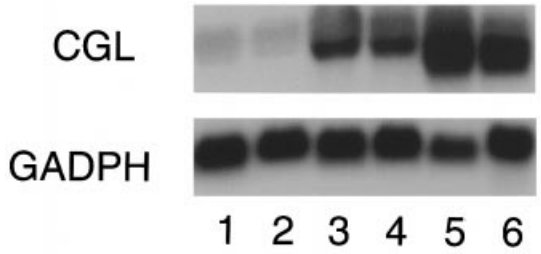

Figure 3 CGL mRNA expression of 293T cells transfected with the two CGL isoforms

RNA (10 μg) was loaded on each lane. Lanes 1 and 2, cells transfected with an empty pcDNA3 vector; lanes 3 and 4, cells transfected with pcDNA-GCL-L; lanes 5 and 6, cells transfected with pcDNA-GCL-S. The equality of the RNA loading was assessed by hybridization with GAPDH.

(GenBank accession number S52784), except for a $G^{87} \rightarrow A$ mutation, which did not cause any change in amino acid sequence.

The longer form of CGL was found to be the more abundant of the two isoforms in the liver. This is in contrast to earlier reports [10,15], in which the shorter form was either predominant or not found at all. However, the sources of the CGL sequence in those studies were a human liver cDNA library and total RNA from HepG2 liver cells, and the differences may be explained either by artifacts in creating a cDNA library or by aberrant mRNA expression in the malignant cell line.

There was a 1.5-fold increase in CGL activity in pcDNA-CGL-L-transfected 293T cells compared with cells transfected with an empty pcDNA3 vector (Figure 2). Transfection with pcDNA-CGL-S did not cause any increase in activity. Although the change from basal activity was small, it was consistent and reproducible. Efficient transcription of both isoforms in transfected cells was verified by Northern blotting (Figure 3). The CGL mRNA expression of cells transfected with pcDNA-CGL-L and pcDNA-CGL-S was 7- and 11-fold, respectively, in comparison with basal CGL mRNA expression of mock-transfected cells. The β-galactosidase activities of the cells co-transfected with β-galactosidase expression vector were of the same order (results not shown).

The existence of an inactive CGL mRNA splice variant is puzzling. Human lysosomal β-galactosidase [16] and mouse tyrosinase [17] have alternatively spliced gene products that encode catalytically inactive proteins. Similarly, rat cystathionine β-synthase has two alternatively spliced isoforms, which do not possess catalytic activity [18]. Furthermore, these forms are degraded rapidly when expressed in mammalian cells. Since we have no means of detecting CGL protein we cannot confirm whether or not CGL-S protein is expressed in cells.

CGL, cystathionine β-lyase and cystathionine γ-synthase (CGS) are members of the γ-family of pyridoxal 5′-phosphate (PLP)-dependent enzymes [19]. The significant sequence similarity between various members of the family [15] and the structural similarity of *Escherichia coli* cystathionine β-lyase [20] and CGS [21], as well as *Nicotiana tabacum* CGS [22], suggest that CGL will have a similar overall fold to that of a canonical member of the family. The available high-resolution structures thus serve to identify important regions in human CGL.

The known members of the γ-family of PLP-dependent enzymes are tetramers. Each monomer consists of three distinct domains: (i) the N-terminal domain, which is part of the active site of the partner monomer within the active dimer, (ii) a PLP-binding domain carrying most of the catalytically important residues and (iii) the C-terminal domain. Two monomers are in close contact sharing active-site residues and forming an active dimer with two active sites. Two of these dimers then interact to form a dimer of dimers.

The overall structure of GCL-L is likely to resemble that of the other members of the γ-family. However, CGL-S lacks 44 residues, located in the PLP-binding domain, including catalytically indispensable residues Asp-187 and Asn-188, which are conserved in all members of the family [15,20]. This suggests that CGL-S is inactive [15]. Yet residues contributing to the active site of the partner subunit as well as the residues participating in the intra- and interdimer interactions remain intact. Therefore the lost fragment may not interfere with the activity of the partner subunit nor affect the tetramer formation. However, the missing fragment will necessitate major movements of the secondary structure elements, which may result in a considerable alteration of the tertiary structure.

It has been postulated that the combination of the two CGL subunits would be a means to regulate the activity of the tetramer [10]. In our study, transfection with CGL-S neither increased nor decreased the basal CGL activity. This finding does not support the hypothesized regulatory function of CGL-S. If CGL-S protein is present in the cell, it may bind to CGL-L, but leave the active site intact. This would mean that in CGL-S overexpressing cells the number of active sites, and hence the CGL activity, stays constant.

Splicing

Amplification of the 5′ intron from genomic DNA resulted in an ≈ 2.3-kb PCR fragment, in which the first 93 nt from the 5′ end as well as 71 nt from the 3′ end were similar to the reported

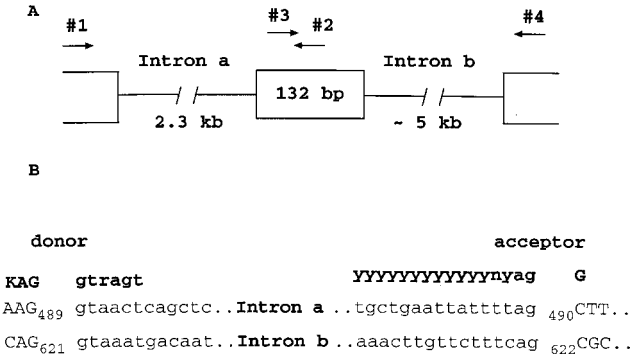

Figure 4 CGL gene at the site of splice variation

(**A**) Exon–intron structure of the human CGL gene at the site of splice variation. Exons are indicated as empty boxes and introns as thin lines. Primer positions are indicated by arrows numbered as follows: #1 and #2, forward and reverse primers for amplification of intron a; #3 and #4, forward and reverse primers for amplification of intron b. (**B**) Exon–intron junctions of the human CGL gene. Sequences flanking exon–intron junctions are aligned with consensus splicing sequences [23]. K indicates A or C, r indicates either purine (A or G), y indicates either pyrimidine (C or T), and n indicates any base. Exon sequences are in upper-case letters and intron sequences are in lower-case letters. The intron positions in cDNA are shown as subscript numbers.

cDNA sequence [10]. A BLAST sequence similarity search of the GenBank database did not show similarity with any other known nucleotide sequences. At the putative exon–intron boundaries, consensus U2-type GT-AG 5′ donor and acceptor sequences [23] were found (Figure 4).

Of the 5-kb DNA fragment resulting from the amplification of the 3′ intron, 108 nt at the 5′ end and 41 nt from the 3′ end were similar to the reported cDNA sequence and to no other sequence found in a BLAST search. The putative exon--intron junctions were consensus sequences (Figure 4) [24]. The fragment missing in CGL-S appears to be coded by a single exon as the two genomic PCR fragments were overlapping.

Expression of CGL during human development

CGL activity was only detected in adult liver tissue (68 ± 9 nmol of cysteine/h per mg of protein), whereas activities in fetal, premature and full-term neonatal liver tissues were undetectable. This is in accordance with the earlier reports [5,6], in which no activity was detected in the developing human liver. In contrast, mRNA expression was detected from the 19th gestational week onwards (Figure 5A), and the mRNA levels were comparable with those of adult liver samples. The expression of the two mRNA forms varied in parallel ($r = 0.94$, $P < 0.001$). Since in the ribonuclease-protection assay the length and the specific activity of the probe affects the intensity of the signal, exact quantification of mRNA is not possible by this method. However, the results of the RT-PCR (Figure 5B) suggest that the longer form is more abundant in the liver throughout development.

There are several possible explanations for the discrepancy between CGL mRNA expression and enzyme activity. Firstly, it is possible that the lack of CGL activity is due to an inhibitor present in the crude extracts of fetal liver. The homogenates were gel-filtered to remove any small molecular-mass inhibitors. In earlier studies [5,6], fetal liver extracts were incubated with crude extracts of rat liver and no change of the rat CGL activity was detected. Thus the presence of an inhibitor in fetal liver is unlikely.

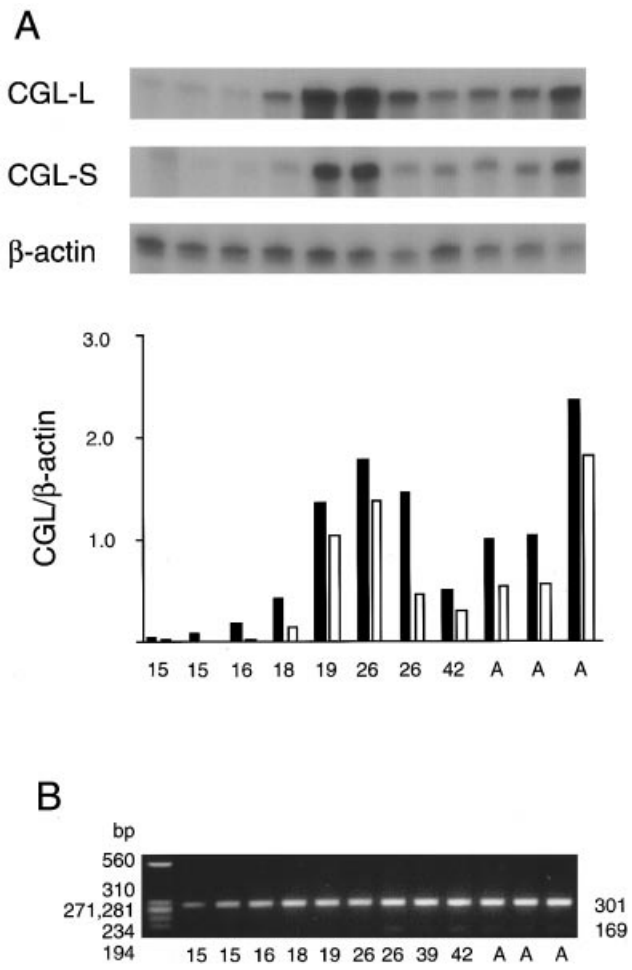

Figure 5 mRNA levels of CGL in human liver during development

(**A**) CGL-L (black bars) and CGL-S (white bars) mRNA levels were studied by ribonuclease-protection assay and quantified relative to β-actin signal. The original autoradiographs of CGL-L, CGL-S and β-actin are shown above each lane. (**B**) mRNA expression was studied by RT-PCR. Line 1, λ-*Hin*dIII and ϕX174 RF-*Hae*III digests. On both panels, numbers indicate weeks of gestation for fetuses and newborns; A, adult.

Secondly, fetal liver may produce CGL that is catalytically inactive. However, polyclonal antiserum raised against purified adult human liver CGL failed to detect immunogenic CGL protein in human fetal liver extracts [25]. Although it is possible that fetal liver produces CGL protein with different immunological properties from the mature enzyme, this is not likely.

Finally, the most plausible explanation for the discrepancy between CGL mRNA levels and enzyme activity is post-transcriptional regulation of CGL gene expression. Translational regulation is a common control mechanism of gene expression during development. In the liver, the activity of many enzymes increases perinatally [26]. Although a majority of these enzymes is regulated at the level of transcription, a number of enzymes have been shown to be regulated post-transcriptionally [27–30]. The postnatal increase in CGL activity may be caused by enhanced mRNA translation or increased protein stability, or both. Translation can be regulated by modulating the rate of translational initiation, by sequestering mRNAs in translationally inaccessible messenger ribonucleoprotein particles (‘ribosomal masking’) or by regulating the length of the poly(A)$^+$

tail [31]. This study does not allow us to discern which mechanism of regulation is operative, nor is it possible to obtain detailed information of temporal changes in mRNA expression and enzyme activity during development from human samples.

In conclusion, we have shown that transfection with the longer CGL isoform increased CGL activity, while the activity of the cells transfected with the shorter form did not differ from the basal activity of 293T cells. In human, CGL activity is absent from fetal liver, whereas both mRNA forms are expressed abundantly. CGL mRNA forms appear to be splice variants.

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