Identification of an important cysteine residue in human glutamate–cysteine ligase catalytic subunit by site-directed mutagenesis

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Glutamate–cysteine ligase (GLCL) catalyses the rate-limiting step in glutathione biosynthesis. To identify cysteine residues in GLCL that are involved in its activity, eight conserved cysteine residues in human GLCL catalytic subunit (hGLCLC) were replaced with glycine residues by PCR-based site-directed mutagenesis. Both recombinant hGLCLC and hGLCL holoenzyme were expressed and purified with a baculovirus expression system. The activity of purified hGLCL holoenzyme with the mutant hGLCLC-C553G was 110 ± 12 $\mu\text{mol/h}$ per mg of protein compared with 370 ± 20 $\mu\text{mol/h}$ per mg of protein for the wild-type. Holoenzymes with hGLCLC-C52G, -C248G, -C249G, -C295G, -C491G, -C501G or -C605G showed activities similar to the wild

type. The K_m values of hGLCL containing hGLCLC-C553G were slightly lower than those of the wild type, indicating that the replacement of cysteine-553 with Gly in hGLCLC did not significantly affect substrate binding by the enzyme. hGLCLC-C553G was more easily dissociated from hGLCLR than the wild-type hGLCLC. GLCL activity increased by 11 % after hGLCLC-C553G was incubated with an equimolar amount of purified hGLCL regulatory subunit (hGLCLR) at room temperature for 30 min, but increased by 110 % after wild-type hGLCLC was incubated with hGLCLR for 10 min. These results indicate that cysteine-553 in hGLCLC is involved in heterodimer formation between hGLCLC and hGLCLR.

INTRODUCTION

Glutamate–cysteine ligase (GLCL, EC 6.3.2.2; γ -glutamyl-cysteine synthetase) catalyses the rate-limiting step in glutathione biosynthesis. GLCL is a heterodimer comprising regulatory (GLCLR) and catalytic (GLCLC) subunits. GLCLC alone catalyses the formation of L- γ -glutamyl-L-cysteine, but the activity of GLCLC is increased by GLCLR [1,2]. The highest GLCL activity and glutathione concentrations are found in cells co-transfected with equal amounts of both subunit cDNA clones [3]. Increased expression of both hGLCLC and hGLCLR genes is seen in cells exposed to β -naphthoflavone, a potent inducer of phase II enzymes [4]. It is therefore apparent that the balanced expression of both hGLCLC and hGLCLR, which are located on chromosomes 1p21 and 6 respectively [5,6], is important for enzyme function.

Both rat and human GLCL activities decrease after incubation with reducing agents, indicating that cysteine residues in GLCL might be involved in disulphide-bond formation between GLCLR and GLCLC [2,7,8]. A thiol group in the GLCL holoenzyme reacts with cystamine, which results in a loss of activity [9,10]. None of the cysteine residues that might be involved in disulphide-bond formation nor the cysteine residues present as the active-site thiol group in GLCL have been identified. An analysis of hGLCLC amino acid sequence translated from the nucleotide sequence revealed the presence of 14 cysteine residues in the hGLCLC protein [11]. Numerous studies have shown that conserved cysteine residues are involved in the catalytic activity of a range of enzymes [12–15]. Therefore the conserved cysteine residues in GLCLC from different species might be important for GLCL activity [11,16–19].

In the present studies the role of conserved cysteine residues was examined by analysing the catalytic activity of hGLCLC mutants in which the conserved cysteine residues were replaced with glycine residues by site-directed mutagenesis. The activity of purified hGLCL holoenzyme with a mutated hGLCLC (hGLCLC-C553G) was 70 % less than that of the wild-type hGLCL holoenzyme. hGLCLC-C553G was more easily dissociated from hGLCLR than the wild-type hGLCLC. When hGLCLC-C553G was mixed with hGLCLR, the rate of increase in activity was slower than when the wild-type hGLCLC was mixed with hGLCLR. These results indicate that Cys-553 in hGLCLC was involved in the interaction of hGLCLC with hGLCLR.

EXPERIMENTAL

Sequence analysis

Multiple GLCL amino acid sequence alignment was performed on an Indigo2 computer (Silicon Graphics, Mountain View, CA, U.S.A.) with CLUSTAL V software [20]. Surface probability and antigenic index were analysed on a PC computer with GeneRunner version 3.04 (Hastings Software, Hastings, NY, U.S.A.).

Site-directed mutagenesis

Site-directed mutagenesis was performed by the method of Weiner et al. [21]. PCR reactions (20 μl) contained 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl, pH 8.8, 2 mM MgSO_4 , 0.1 % (v/v) Triton X-100, 0.1 mg/ml BSA, 20 ng of hGLCLC in the pFastBac HT_a vector (pHT-GLCLC) [8], each deoxynucleotide triphosphate at 250 μM , 0.5 unit of cloned *pfu* DNA

Abbreviations used: DTT, dithiothreitol; GLCL, glutamate–cysteine ligase; hGLCL, human GLCL; hGLCLC, catalytic subunit of hGLCL; hGLCLR, regulatory subunit of hGLCL.

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Table 1 PCR primers and their location in hGLCLC

The location of oligonucleotides in the sense strand of hGLCLC is based on the published hGLCLC sequence [11]. The mutated nucleotides are in lower-case letters.

Mutant	Oligonucleotide sequence	Location in hGLCLC (nt)
GLCLC-C52G	CAGATTAGGcGTCCTGGGTTcACAC	536–561
GLCLC-C248G	GGAATGGGCAATgGCTGTCTCCAGG	822–846
GLCLC-C249G	GGGCAATTGcGgTCTCCAGGTGAC	827–850
GLCLC-C295G	CAGACATTGATgTGCCTGGGGAGTG	964–989
GLCLC-C491G	CAGGAAAGATATTgGCAAGGTGGC	1551–1575
GLCLC-C501G	CAGTGGTGGATGGTgTGGCAAGGC	1579–1603
GLCLC-C553G	GGACACCAGAgGTAGTATTCTGAAC	1733–1757
GLCLC-C605G	GCCTTATTTTGAAGgGTAAcCAATTGC	1892–1918

polymerase (Stratagene, La Jolla, CA, U.S.A.) and a pair of complementary primers (Table 1). PCR primers were designed to contain a single nucleotide change that resulted in the change of the amino acid codon from cysteine to glycine. Reactions were overlaid with 30 μ l of mineral oil. PCR was performed on a DNA Thermal Cycler 480 (Perkin–Elmer, Branchburg, NJ, U.S.A.) for 20 cycles (each cycle consisting of 30 s at 60 or 65 °C, 14 min at 72 °C, 30 s at 95 °C) after denaturation at 95 °C for 2 min. After PCR, 10 μ l of the reaction mixture was analysed on 0.5% agarose gel. Ethanol was added to the remainder of reaction mixture, and the precipitated DNA was digested with 5 units of *DpnI* (Promega, Madison, WI, U.S.A.) in a 10 μ l reaction mixture at 37 °C for 3 h. Competent DH10B cells (Gibco BRL) were transformed by *DpnI*-digested DNA with an Electro Cell Manipulator 600 (BXT, San Diego, CA, U.S.A.). Plasmid DNA was sequenced by dideoxynucleotide sequencing. Recombinant baculovirus DNA was obtained by transformation of Max efficiency DH10Bac competent cells (Gibco BRL) with pHT-GLCLC DNA, in accordance with the manufacturer's directions.

Cells

Sf9 insect cells (Gibco BRL) were grown in Sf-900 II SFM serum-free insect medium (Gibco BRL). Cells were maintained in suspension cultures at 28 °C with constant agitation at 125 rev./min.

Transfections

Recombinant baculovirus DNA (2–3 μ g) was mixed with 200 μ l of Sf-900 II SFM and 6 μ l of CellFectin Reagent (Gibco BRL). The mixture was incubated at room temperature for 45 min and then added to 10⁶ cells with 0.8 ml of Sf-900 II SFM. After 5 h, the medium was removed and 2 ml of Sf-900 II SFM was added. Recombinant baculoviruses were harvested 72 h after transfection, then stored at 4 °C. Recombinant hGLCLR and hGLCLC baculoviruses were mixed in a 1:1 ratio for the expression of hGLCLC holoenzyme.

Purification of hGLCLR and hGLCLC

Cells were harvested 48–72 h after infection by recombinant baculovirus and centrifuged at 100 *g* for 10 min. The cells were lysed with lysis buffer [50 mM Tris/HCl (pH 8.5)/1 mM PMSF/1% (v/v) Nonidet P40]. After centrifugation at 13000 *g* for 10 min, the supernatant from the cell lysate was mixed with nickel nitrilotriacetate (Ni/NTA) resin (Qiagen, Chatsworth,

CA, U.S.A.) pre-equilibrated with buffer A [20 mM Tris/HCl (pH 8.5)/500 mM KCl/20 mM imidazole/10% (v/v) glycerol], and the suspension was incubated at 4 °C for 30 min. The Ni/NTA resin was sedimented by centrifugation at 1000 *g* for 2 min and suspended in buffer A. The adherent proteins were eluted from the Ni/NTA resin with 20 mM Tris/HCl (pH 8.5 at 4 °C)/100 mM KCl/100 mM imidazole/10% (v/v) glycerol. Protein concentrations were determined by the Bradford method with protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.). Proteins (0.5 μ g) in sample buffer [60 mM Tris/HCl/145 mM mercaptoethanol/10% (v/v) glycerol/1% SDS (pH 6.8)] were heated in a boiling-water bath for 5 min, except where indicated, and were resolved by SDS/PAGE [7.5% (w/v) gel]. Gels were stained with Coomassie Brilliant Blue.

Gel-filtration chromatography

Samples were analysed on a HPLC Bio-Sil SEC-250 gel-filtration column (Bio-Rad) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. The absorbance of the eluate was measured at 280 nm with a LC-235 diode-array detector (Perkin–Elmer, Norwalk, CT, U.S.A.). The gel-filtration column was calibrated with protein molecular-mass standards (Bio-Rad).

GLCL activity assay

GLCL activity was determined by measuring the rate of ADP formation spectrometrically with a coupled assay with pyruvate kinase and lactate dehydrogenase [2,10]. The reaction mixtures (0.5 ml) contained 100 mM Tris/HCl, pH 8.0, 150 mM KCl, 20 mM MgCl₂, 0.2 mM NADH, 5 mM ATP, 2 mM sodium phosphoenolpyruvate, 2 mM Na₂EDTA, 10 mM sodium L-glutamate, 10 mM sodium L- α -aminobutyrate, 2 units of pyruvate kinase (rabbit muscle type II; Sigma, St. Louis, MO, U.S.A.) and 10 units of lactate dehydrogenase (bovine heart type III; Sigma). Reaction mixtures were incubated at 37 °C and were initiated by the addition of purified recombinant hGLCL. The change in absorbance at 340 nm was measured with a Beckman spectrophotometer (Model DU-45) over 5 min.

Kinetic analysis

L-Glutamate concentrations ranging from 0.1 to 10 mM were used to determine the K_m of L-Glu; the L- α -aminobutyrate and ATP concentrations were 10 and 5 mM respectively. L- α -Aminobutyrate or L-cysteine concentrations ranging from 0.1 to 10 mM were used to determine the K_m of L- α -aminobutyrate or L-cysteine; the L-glutamate and ATP concentrations were 10 and 5 mM respectively. The K_m and V_{max} values were calculated with the EZ-Fit computer program, version 2.0 (Perrella Scientific, Springfield, PA, U.S.A.).

CD spectroscopy

The elution buffer containing imidazole and KCl interfered with the measurement of CD. Therefore the elution buffer was exchanged with 10 mM sodium phosphate, pH 7.0, from purified proteins with Econo-Pac 10DG columns (Bio-Rad). CD spectra of approx. 1 μ M protein in 10 mM sodium phosphate, pH 7.0, were recorded from 260 to 180 nm at 20 °C on a Jasco J-710 spectropolarimeter (Jasco Corp., Tokyo, Japan). Protein secondary structures were predicted with J-700 for Windows Secondary Structure Estimation, version 1.10.00 (Jasco Corp.).

RESULTS

There are 14 cysteine residues in hGLCLC [11]. Amino acid sequence alignment of three published mammalian GLCLC sequences [19,22,23] showed that all 14 cysteine residues in hGLCLC were conserved. To identify the potentially important cysteine residues in hGLCLC, the amino acid sequences of GLCL from *Schizosaccharomyces pombe* [17], *Trypanosoma brucei* [18] and *Escherichia coli* [16] were compared. GLCL genes from *Arabidopsis thaliana* [24] and *Thiobacillus ferrooxidans* [25] have low identity (15–18%) with mammalian, yeast and *E. coli* GLCLs [16], and their sequences were not included in the sequence alignment. Eight cysteine residues were conserved, at least, in four species. The locations of the conserved cysteine residues in hGLCLC are indicated in Table 1.

Mutations were induced into hGLCLC in pFastBac HT vector [8] with a pair of complementary primers (Table 1). After PCR reactions, products of approx. 7 kb DNA were visible on agarose gels after electrophoresis, as expected (results not shown). The PCR products were digested with *DpnI* and were used to transform competent bacterial cells. Colonies (20–200) were obtained from each transformation of competent bacterial cells. Clones containing site-directed mutations were verified by dideoxy DNA sequencing analysis (results not shown). No other mutations were found in the region (approx. 200 bp) including the mutated site. The sequence of the entire coding region of GLCLC in the hGLCLC-C553G cDNA clone was determined, and no spurious mutations were found (results not shown).

Recombinant baculoviruses were obtained from all pHT-hGLCLC mutants after transfection of insect cells with recombinant baculovirus DNA (Table 2). hGLCLC proteins expressed in cells infected by recombinant baculoviruses were purified. The purified proteins appeared homogeneous on SDS/PAGE and showed molecular masses of approx. 75 kDa (Figure 1A). All purified hGLCLC mutants showed GLCL activity (Table 2). The GLCL activities of hGLCLC-C52G, -C248G, -C249G, -C295G, -C501G and -C605G were significantly lower than that of the wild-type hGLCLC, whereas the activities of mutants hGLCLC-C491G and -C553G were comparable with that of the wild-type hGLCLC. To obtain hGLCL holoenzyme, insect cells were co-infected with equal amounts of recombinant hGLCLC and recombinant wild-type hGLCLR baculoviruses. hGLCL holoenzyme was purified to homogeneity from infected cells (Figure 1B). The holoenzymes were dissociated into two subunits under

Figure 1 Electrophoresis of purified recombinant hGLCL

Purified hGLCL was analysed by SDS/PAGE [7.5% (w/v) gel]; the gel was stained with Coomassie Brilliant Blue. (A) Purified recombinant hGLCLC. Lane M, molecular-mass markers; lanes 1–9, 0.5 μ g of purified hGLCLC from cells infected with recombinant hGLCLC-C52G, -C248G, -C249G, -C295G, -C491G, -C501G, -C553G, -C605G or wild-type hGLCLC baculoviruses respectively. (B) Purified recombinant hGLCL holoenzymes. Lanes 1–9, 0.5 μ g of purified hGLCL holoenzyme from cells co-infected with recombinant wild-type hGLCLR baculovirus along with recombinant wild-type hGLCLC, hGLCLC-C52G, -C248G, -C249G, -C295G, -C491G, -C501G, -C553G or -C605G baculoviruses respectively.

Table 2 Enzymic activity of wild-type and mutated hGLCL and wild-type and mutated hGLCLC + hGLCLR holoenzymes

Activity was determined as described in the Experimental section. Data are means $\pm$ S.D. ($n = 3$ –5). *Significant difference ($P < 0.05$; ANOVA) between GLCL activity in mutants and in the wild-type proteins.

hGLCLC	hGLCLC activity (μ mol/h per mg of protein)	hGLCL holoenzyme	hGLCL holoenzyme activity (μ mol/h per mg of protein)
hGLCLC	69 $\pm$ 26	hGLCLC + hGLCLR	370 $\pm$ 20
hGLCLC-C52G	10 $\pm$ 0*	hGLCLC-C52G + hGLCLR	400 $\pm$ 20
hGLCLC-C248G	10 $\pm$ 0*	hGLCLC-C248G + hGLCLR	340 $\pm$ 26
hGLCLC-C249G	7 $\pm$ 4*	hGLCLC-C249G + hGLCLR	280 $\pm$ 40
hGLCLC-C295G	7 $\pm$ 4*	hGLCLC-C295G + hGLCLR	320 $\pm$ 6
hGLCLC-C491G	38 $\pm$ 10	hGLCLC-C491G + hGLCLR	420 $\pm$ 12
hGLCLC-C501G	29 $\pm$ 5*	hGLCLC-C501G + hGLCLR	420 $\pm$ 6
hGLCLC-C553G	55 $\pm$ 11	hGLCLC-C553G + hGLCLR	110 $\pm$ 12*
hGLCLC-C605G	23 $\pm$ 11*	hGLCLC-C605G + hGLCLR	310 $\pm$ 15

reducing conditions after SDS/PAGE. All purified hGLCL holoenzymes exhibited apparent molecular masses of 114 kDa by gel-filtration chromatography (results not shown), which confirmed the formation of the hGLCLC and hGLCLR heterodimer. Although the replacement of cysteine with glycine affected the activities of hGLCLC-C52G, -C248G, -C249G, -C295G, -C501G and -C605G, the activities of hGLCLC-C52G, -C248G, -C249G, -C295G, -C501G and -C605G holoenzymes with hGLCLR were comparable with that of the wild-type holoenzyme (Table 2), indicating that hGLCLR restored the activities of mutated hGLCLCs. The hGLCLC-C553G + hGLCLR holoenzyme exhibited significantly lower enzymic activity (30%) than the hGLCL holoenzymes that contained the wild-type hGLCLC or other mutated hGLCLCs, indicating that Cys-553 in hGLCLC is important for the activity of hGLCL holoenzyme. The K_m values of hGLCLC-C553G + hGLCLR holoenzyme for L-glutamate and L- α -aminobutyrate were slightly higher than those of the wild type (Table 3). Therefore the replacement of Cys-553 residues with glycines had little effect on the K_m for L-glutamate and L- α -aminobutyrate of hGLCL holoenzyme. The wild-type holoenzyme showed a higher K_m for cysteine than did the hGLCLC-C553G + hGLCLR holoenzyme.

Cystamine interacts with a thiol group in rat and human GLCL holoenzymes that results in a complete loss of GLCL activity [9,26,27]. In the present studies it was also observed that hGLCLC-C553G + hGLCLR holoenzyme as well as the wild-type hGLCL holoenzyme (as a control) lost activity after incubation with 0.2 mM cystamine and 2 mM ATP at 4 $^{\circ}$ C for

Table 3 K_m and V_{max} values of recombinant hGLCL holoenzyme

The K_m and V_{max} values of recombinant hGLCL holoenzyme for L-glutamate, L- α -aminobutyrate and L-cysteine were determined from the reaction velocities in the presence of each variable (0.1–10 mM) and fixed (10 mM) substrate with 5 mM ATP. *Significant difference ($P < 0.05$, ANOVA) between wild-type hGLCL holoenzyme and hGLCLC-C553G + wild-type hGLCLR.

hGLCL holoenzyme	K_m (mM)			V_{max} (mmol/min per mg)		
	L-Glu	L- α -Aminobutyrate	L-Cys	L-Glu	L- α -Aminobutyrate	L-Cys
Wild-type	0.7 ± 0.1	3.6 ± 0.4	$0.8 \pm 0.1^*$	3.6 ± 0.2	4.2 ± 0.6	$11.2 \pm 0.5^*$
hGLCLC-C553G	0.9 ± 0.2	5.0 ± 1.2	0.5 ± 0.1	2.3 ± 0.1	3.1 ± 0.8	1.8 ± 0.1

Figure 2 CD spectra of wild-type hGLCLC and hGLCLC-C553G

CD spectra of purified protein in 10 mM sodium phosphate (pH 7.0) were recorded from 260 to 180 nm at 20 °C on a Jasco J-710 spectropolarimeter. (A) Wild-type hGLCLC; (B) hGLCLC-C553G.

90 min (results not shown). Therefore Cys-553 in hGLCLC is not likely to be the putative active-site thiol in hGLCLC.

CD spectroscopy is a valuable tool for the study of protein conformations and for assessing the secondary structure of proteins [28]. The secondary structure of the wild-type hGLCLC

Figure 3 Non-reducing SDS/PAGE of purified recombinant wild-type hGLCL and hGLCLC-C553G + hGLCLR holoenzymes

Purified hGLCL was analysed by SDS/PAGE [7.5% (w/v) gel]; the gel was stained with Coomassie Brilliant Blue. Lane M, molecular-mass markers; lane 1, 1 μ g of purified wild-type hGLCL holoenzyme with loading buffer [1% (w/v) SDS/10% (v/v) glycerol/60 mM Tris/HCl (pH 6.8)]; lane 2, 1 μ g of purified wild-type hGLCL holoenzyme was incubated with loading buffer at 100 °C for 5 min; lane 3, 1 μ g of purified hGLCLC-C553G + hGLCLR holoenzyme with loading buffer; lane 4, 1 μ g of purified hGLCLC-C553G + hGLCLR holoenzyme was incubated with loading buffer at 100 °C for 5 min.

and mutant hGLCLC-C553G were estimated from the CD spectra of the proteins over the absorbance range 260–180 nm (Figure 2). The compositions (%) of predicted α -helices and β -sheets in the mutant hGLCLC-C553G were 28.3 ± 2.9 and 39.6 ± 5.3 respectively, which was similar to those of the wild-type (27.8 ± 8.6 and 40.3 ± 3.3 respectively), indicating that the overall structure of hGLCLC was not altered by the C553G mutation.

The C553G mutation resulted in a significant decrease in hGLCLC-C553G + hGLCLR holoenzyme activity. The effect of this mutation was therefore studied in more detail. Non-reduced, purified recombinant wild-type hGLCL and hGLCLC-C553G + hGLCLR holoenzymes were separated by SDS/PAGE with or without heating (Figure 3). Most of the wild-type hGLCL was present as the holoenzyme (Figure 3, lane 1). Considerable dissociation into hGLCLC and hGLCLR was seen after heating (Figure 3, lane 2). In contrast, purified hGLCLC-C553G + hGLCLR holoenzyme was easily dissociated into hGLCLC and hGLCLR subunits (Figure 3, lane 3), and the increased dissociation of the mutated holoenzyme observed after heating (Figure 3, lane 4) might have been due to other interactions between hGLCLC and hGLCLR.

If hGLCLC-C553G does not associate properly with hGLCLR, the increase in GLCL activity seen after incubating equimolar amounts of wild-type hGLCLC and hGLCLR might be slower than when hGLCLC-C553G is incubated with an equimolar amount of the wild-type hGLCLR. Because the increase in activity after incubation of wild-type hGLCLC and hGLCLR was slow at 4 °C (results not shown), the change in

Figure 4 Time-dependent activation of hGLCLC by hGLCLR

Purified recombinant hGLCLC (1 μ g) was incubated with 0.4 μ g of purified recombinant hGLCLR for the indicated durations. GLCL activity is expressed as a percentage of the activity of 1 μ g of purified recombinant hGLCLC with 0.4 μ g of hGLCLR activity at 0 min. Zero-time activities with wild-type hGLCLC + hGLCLR (●) and hGLCLC-C553G + hGLCLR (○) were 83 ± 10 and 54 ± 5 μ mol/h per mg of protein respectively. Data are means $\pm$ S.D. ($n = 3$).

Figure 5 Effect of DTT on hGLCLC holoenzyme activity

Purified recombinant wild-type hGLCLC holoenzyme (1 μ g) (open columns) or recombinant hGLCLC-C553G + hGLCLR (1 μ g) (hatched columns) was incubated in the absence or presence of 1 or 10 mM DTT in 50 mM Tris/HCl, pH 8.0, at 4 °C for 30 min. GLCL activity was measured as described in the Experimental section. Control activities for the wild-type hGLCLC and hGLCLC-C553G + hGLCLR were 370 ± 20 and 110 ± 12 μ mol/h per mg of protein respectively. Data are means $\pm$ S.D. ($n = 3$).

activity after incubating hGLCLC with hGLCLR was studied at room temperature for both the wild-type and the mutant hGLCLC holoenzymes. GLCL activity increased to approx. 110% of values at zero time when wild-type hGLCLC was incubated with hGLCLR for 10 min at room temperature (Figure 4); no further increase in activity was seen with longer incubation times. In contrast with the wild-type hGLCLC, GLCL activity increased by 8% and 11% after hGLCLC-C553G was incubated with hGLCLR for 10 and 30 min at room temperature respectively. These results indicate that the interaction of hGLCLR with hGLCLC is time-dependent and that the interaction of hGLCLC with hGLCLR is impaired by the C553G mutation in hGLCLC.

It has been proposed that intermolecular and intramolecular disulphide bonds are present between rat GLCLC and GLCLR [2]. The disulphide bonds seemed to be important for the enzyme's activity because reduced rat GLCLC holoenzyme lost activity [7]. The activities of wild-type hGLCLC and hGLCLC-C553G + hGLCLR holoenzyme were measured after incubation with different concentrations of dithiothreitol (DTT) at room temperature for 30 min. A time- and DTT-concentration-dependent decrease in GLCL activity was observed (results not shown). The decrease in enzyme activity amounted to 14% and 43% for wild-type hGLCLC holoenzyme after incubation with 1 and 10 mM DTT respectively (Figure 5); hGLCLC-

C553G + hGLCLR holoenzyme lost 46% and 56% activity after incubation with 1 and 10 mM DTT respectively. Therefore the hGLCLC-C553G + hGLCLR holoenzyme was more sensitive than the wild-type hGLCLC holoenzyme to the reducing agent.

DISCUSSION

Sequence alignments are used to help to identify conserved residues that are potentially important for enzyme activity [13–15]. Cys-553 is conserved in GLCLCs from three mammalian species and from yeast. Computer-based secondary-structure analysis revealed that Cys-553 was probably located at the end of a loop or on an α -helix. Interestingly, Cys-553 is located in a region with low surface probability, although the antigenic index of the region is high. Mammalian GLCL consists of catalytic and regulatory subunits that are translated from two separate genes [11,19], whereas bacterial and yeast GLCL genes encode a single peptide [16,17]. It would be of interest to determine whether the cysteine residue identified in the present studies is involved in intramolecular interactions in yeast.

PCR-based site-directed mutagenesis is a powerful tool for studying the roles of particular amino acid residues in proteins [12,21]. In the present study, mutants were obtained by PCR of a double-stranded hGLCLC-containing plasmid (pFastBac HT) with a pair of complementary primers containing mutations. This approach does not require the presence of unique restriction sites in the GLCL for the subsequent insertion of a mutated fragment, which allows the generation of mutations at any site. *pfu* DNA polymerase has the lowest observed mutation rate compared with other commercially available thermostable DNA polymerases [29,30] and was used in the present study to limit spurious mutations during amplification. Sequencing of approx. 200 bp regions that included the mutated cysteine residues showed no other mutations in all mutants. Moreover, no spurious mutations were found in the hGLCLC coding region in hGLCLC-C553G. In addition, proteins with the correct molecular masses were expressed from all of the mutant clones.

Cystamine completely inactivates rat GLCL activity by interaction with a thiol group in rat and human GLCL holoenzyme [9,26,27]. The thiol in rat GLCL is thought to be at or near the L-glutamate-binding site [9,31]. In the present study it was observed that hGLCLC-C553G holoenzyme lost activity after incubation with cystamine. The K_m of hGLCLC-C553G + hGLCLR holoenzyme for L-glutamate was slightly lower than that of the wild-type. It is therefore likely that the critical thiol in hGLCLC holoenzyme is not Cys-553 in hGLCLC. The role of redox regulation involving an active-site thiol in GLCL is currently unclear and merits further investigation.

When wild-type hGLCLC and hGLCLR were mixed, hGLCLC activity increased over the first 10 min of incubation. In contrast, little increase in activity was observed when hGLCLC-C553G was mixed with wild-type hGLCLR, indicating that Cys-553 in hGLCLC is important for the interaction of hGLCLC with hGLCLR. Because the K_m values of the wild-type hGLCLC holoenzyme for L-glutamate and L- α -aminobutyrate did not significantly differ from those of hGLCLC-C553G + hGLCLR holoenzyme (Table 3), the higher activity of the wild-type hGLCLC might be attributable to a more favourable interaction between hGLCLC and hGLCLR rather than to the increased binding of substrate. A more favourable interaction between wild-type hGLCLC and hGLCLR compared with that of hGLCLC-C553G and hGLCLR was observed by non-reducing gel electrophoresis. The failure to observe an increase in activity when hGLCLC-C553G was incubated with hGLCLR was also re-

flected in a lower activity of hGLCLC-C553G + hGLCLR holoenzyme activity compared with that of the wild-type hGLCL.

In the wild-type hGLCL holoenzyme, approx. 20 % of the activity is attributable to the hGLCLC subunit. In contrast, approx. 50 % of the activity of the hGLCLC-C553G + hGLCLR holoenzyme is attributable to the hGLCLR-C553G subunit. Because the activities of wild-type hGLCLC and hGLCLC-C553G do not differ, the decreased activity of the hGLCLC-C553G + hGLCLR holoenzyme might be attributable to effects of the C553G mutation in the catalytic subunit on its interaction with the regulatory subunit. In the other mutants studied, the decreased activities of the mutated catalytic subunits were not reflected in a decreased activity of the hGLCL holoenzymes. Although the effects of the mutations on the activities of the catalytic subunits are not well understood, these mutations apparently did not affect the association of the subunits in the holoenzyme because the activities of all holoenzymes except hGLCLC-C553G + hGLCLR did not differ from that of the wild-type holoenzyme.

These results indicate the presence of both intermolecular and intramolecular disulphide bonds in hGLCL. The C553G mutation in hGLCLC might impair intermolecular disulphide-bond formation between hGLCLC and hGLCLR, which might expose intramolecular disulphide bonds and facilitate their reduction: the hGLCLC-C553G + hGLCLR holoenzyme was more sensitive to DTT than was the wild-type hGLCL holoenzyme. Other interactions, such as electrostatic and hydrophobic interactions, have been proposed, and it was shown that the hGLCL holoenzyme is not completely dissociated into hGLCLC and hGLCLR even in the presence of 50 mM DTT [8]. Therefore non-covalent interactions might be important in hGLCLC-C553G and hGLCLR subunit interactions; indeed, the hGLCLC-C553G + hGLCLR holoenzyme migrates as a single protein on gel-filtration chromatography.

The results presented here show that the interaction of hGLCLC with hGLCLR is correlated with GLCL activity. Cys-553 in hGLCLC is apparently important for the interaction of hGLCLC with hGLCLR, perhaps owing to disulphide-bond formation. The activity of the hGLCL holoenzyme decreased after treatment with a reducing agent. Conversely, an increase in the oxidation state of the cell might be associated with oxidation of the disulphide bond and might confer increased activity. Additional cellular processing is apparently required for the interaction of hGLCLC and hGLCLR because the activity of a mixture of wild-type hGLCLC and hGLCLR was only approx. 40 % of that of the wild-type hGLCL holoenzyme purified from cells.

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