

## ORIGINAL INVESTIGATION

Wilfried Kugler · Kathrin Breme · Petra Laspe  
Hilary Muirhead · Christopher Davies · Heinz Winkler  
Werner Schröter · Max Lakomek

## Molecular basis of neurological dysfunction coupled with haemolytic anaemia in human glucose-6-phosphate isomerase (GPI) deficiency

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**Abstract** Glucose-6-phosphate isomerase (GPI) deficiency, an autosomal recessive genetic disorder with the typical manifestation of nonspherocytic haemolytic anaemia, can be associated in some cases with neurological impairment. GPI has been found to be identical to neuroleukin (NLK), which has neurotrophic and lymphokine properties. To focus on the possible effects of *GPI* mutations on the central nervous system through an effect on neuroleukin activity, we analysed DNA isolated from two patients with severe GPI deficiency, one of them with additional neurological deficits, and their families. The neurologically affected patient (GPI Homburg) is compound heterozygous for a 59 A→C (H20P) and a 1016 T→C (L339P) exchange. Owing to the insertion of proline, the H20P and L339P mutations are likely to affect the folding and activity of the enzyme. In the second family studied, the two affected siblings showed no neurological symptoms. The identified mutations are 1166 A→G (H389R) and 1549 C→G (L517V), which are located at the subunit interface. We propose that mutations that lead to incorrect folding destroy both catalytic (GPI) and neurotrophic (NLK) activities, thereby leading to the observed clinical symptoms (GPI Homburg). Those alterations at the active site, however, that allow correct folding retain the neurotrophic properties of the molecule (GPI Calden).

### Introduction

Glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) is a dimeric enzyme that catalyses the reversible interconversion of fructose-6-phosphate and glucose-6-phosphate. GPI may have a further function in addition to its essential role in carbohydrate metabolism in all tissues. A protein, originally called neuroleukin (NLK), was found to have neurotrophic and lymphokine properties (Gurney et al. 1986a, b). Sequencing confirmed that neuroleukin is GPI (Chaput et al. 1988; Faik et al. 1988). GPI deficiency in humans is an autosomal recessive genetic disorder that has the typical manifestation of nonspherocytic haemolytic anaemia of variable clinical expression. It has been reported that severe GPI deficiency can be associated with hydrops fetalis, immediate neonatal death and neurological impairment (Whitelaw et al. 1979; Schröter et al. 1985; Eber et al. 1986; Ravindranath et al. 1987; Shalev et al. 1993). Although several GPI variants in humans have been defined in terms of electrophoretic mobility, thermal stability, enzyme activity, and peptide mapping, at the nucleotide level only 22 point mutations have been described to date (reviewed in Beutler et al. 1997; Huppke et al. 1997). We analysed the molecular basis of severe GPI deficiency in two patients, one of them with additional neurological impairment, and their families. Four novel missense mutations (59C, 1016C, 1166G, 1549G) were detected. On the basis of our results we present a hypothesis to explain the relationship between the mutations, the biochemical alterations and the differing clinical manifestations.

W. Kugler · K. Breme · P. Laspe · W. Schröter · M. Lakomek (✉)  
Universitäts-Kinderklinik, Robert-Koch-Strasse 40,  
D-37075 Göttingen, Germany  
Tel.: +49-551-39-6214, Fax: +49-551-39-6231

H. Muirhead · C. Davies  
Department of Biochemistry, University of Bristol,  
University Walk, Bristol BS8 1TD, UK

H. Winkler  
Max-Planck-Institut für Biophysikalische Chemie,  
Am Fassberg,  
D-37077 Göttingen, Germany

### Materials and methods

#### Patients

The detailed clinical history, haematological data and enzyme characteristics of patient I (GPI Homburg), his mother and family II (GPI Calden) have been described previously (Schröter et al. 1985; Eber et al. 1986; Neubauer et al. 1990). They were confirmed recently when

**Table 1** Selected haematological, biochemical and molecular genetic parameters of the affected patients and their heterozygous family members. (f female, m male, G-6-P glucose-6-phosphate, GPI glucose-6-phosphate isomerase)

Subject	Haematological data		Biochemical data			Genetic data	
	Hb (g/dl)	Reticulocytes (%)	GPI activity (IU/g Hb)	Stability <sup>a</sup> (%)	G-6-P (μmol/ml RBC)	Nucleotide change	Structural change
Patient I (propositus)	13.3	17.5	3.5	53	0.143	A→C at 59 T→C at 1016	His→Pro at 20 Leu→Pro at 339
Mother I	15.0	2.1	31	73	0.035	T→C at 1016	Leu→Pro at 339
Patient II/1 (proposita)	11.0	20.0	12	50	0.061	A→G at 1166 C→G at 1549	His→Arg at 389 Leu→Val at 517
Patient II/2 (propositus)	10.5	55.0	14	50	0.067	A→G at 1166 C→G at 1549	His→Arg at 389 Leu→Val at 517
Father II	14.3	2.0	32	86	0.027	C→G at 1549	Leu→Val at 517
Mother II	13.8	2.4	27	79	0.021	A→G at 1166	His→Arg at 389
Reference values	f 12–16 m 14–18	0.5–1.5	50±10.5	94±12	0.026–0.043		

<sup>a</sup> Percentage of activity after 60 min incubation at 45°C

**Table 2** Restriction endonuclease verification of the glucose-6-phosphate isomerase (*GPI*) mutations identified. (*PCR* polymerase chain reaction)

Mutation	Patient	Enzyme	Fragment size (bp)		
			PCR	Normal	Mutant
59A→C	I	<i>Ban</i> II	155	155	92, 63
1016T→C	I	<i>Bbv</i> I	352	118, 98, 59, 31, 24, 22	149, 98, 59, 24, 22
1166A→G	II/1, II/2	<i>Sph</i> I	237	144, 93	237
1549C→G	II/1, II/2	<i>Alu</i> I	116	79, 25, 12	91, 25

the patients and their families were admitted to our hospital for further detailed investigation. In contrast to earlier findings GPI Homburg turned out to be heat labile like most of the other GPI variants. Representative data are shown in Table 1.

#### Conventions

The translation-initiating ATG will be referred to as codon/amino acid no. 1 and the corresponding adenine as nucleotide no. 1. Reference sequences for *GPI/NLK* are GenBank accession nos. S81084 and K03515.

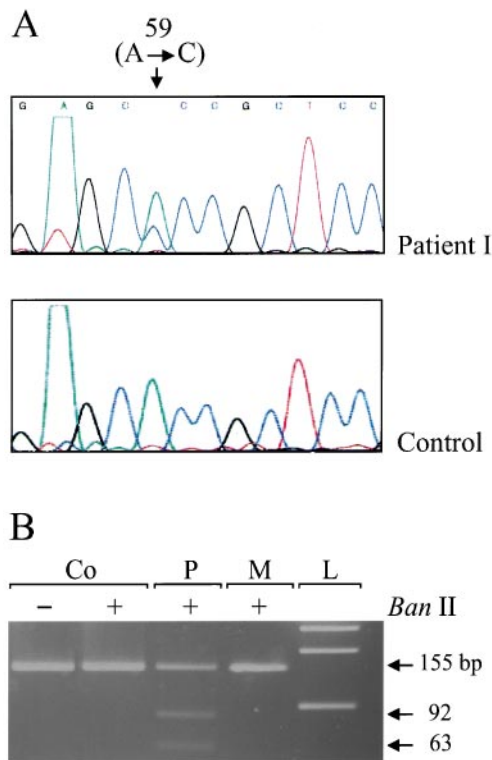
#### Primers

Intron primer pairs sufficiently distant from the intron/exon borders were designed to amplify the whole coding region of the human *GPI*

gene. We used the forward and reverse primers suggested by Xu et al. (1995) and as the reverse primer for exons 1 and 18, respectively, the following (5'→3'): TCCAGACCCCAGGCCCGCGT (1R), ACAAGCTTATACCTCCTCCGTGGCATCT (18R). The sequences of sense (s) and antisense (as) primers (5'→3') for the polymerase chain reaction (PCR) preceding restriction analysis were as follows: CGCGTCTCACTCAGTGTTACC (s), CTGAAGTGGTTGAAGCGGTC (as) for *Ban*II digestion (exon 1), primers for exon 12 (*Bbv*I) and exon 13 (*Sph*I) as suggested by Xu et al. (1995), and GGCTCAGG-GATTCAGTAGCAACG (s), GCTCAGGCTCTATTTTCT (as) for *Alu*I digestion (exon 18).

#### Direct sequencing of amplified genomic DNA and mutation analysis

EDTA-blood samples were obtained from the patients and the family members, and from 60 unrelated blood donors as controls. Genomic DNA was prepared from an aliquot of the whole-blood sample using

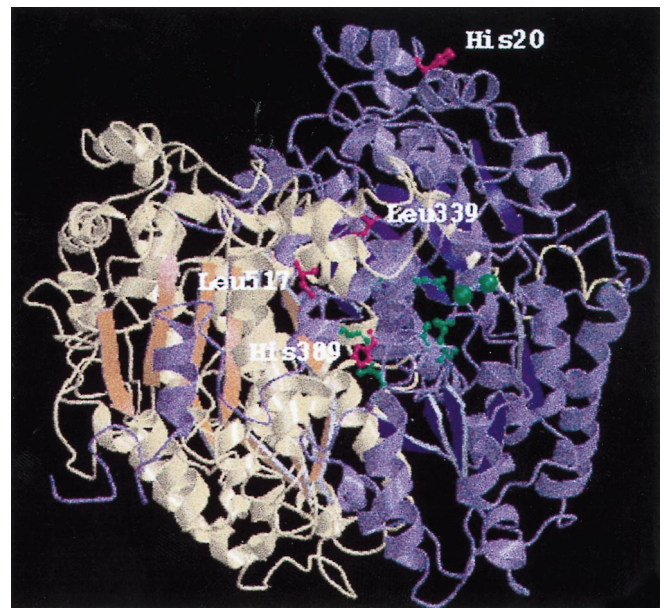


**Fig. 1** **A** Sequence analysis of exon 1 amplified from genomic DNA of patient I (GPI Homburg) and a control. An A→C transversion at nucleotide 59 causes a His→Pro substitution at codon 20. **B** Restriction-digestion analysis of the genomic region around the exon 1 mutation in patient I and his mother. Polymerase chain reaction products of 155 bp were digested with *Ban*II and analysed on a 4% NuSieve 3:1 agarose gel. There is no *Ban*II site in the sequence of the normal control and the patient's mother (*Co*, *M*). However, the mutation at nucleotide 59 creates a *Ban*II site, providing fragments of 92 and 63 bp in addition to the wild-type fragment from patient I (*P*). (*L* 100-bp ladder, +, -, presence and absence of the enzyme during incubation)

QIAamp spin columns (Qiagen, Hilden, Germany). Each exon of the human *GPI* gene was amplified from genomic DNA by PCR using intron primer pairs. The thermal profile for all reactions was denaturation at 90°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 1 min 30 s. The 35 cycles were preceded by a denaturation step at 95°C for 5 min and immediately followed by a final extension at 72°C for 5 min. The double-stranded PCR products were purified with QIAquick spin columns (Qiagen, Hilden, Germany), and 60 ng of the purified DNA was sequenced on an ABI Prism 373A DNA Sequencer by using Amplitaq DNA Polymerase (Perkin-Elmer, Weiterstadt, Germany) FS dye terminator cycle sequencing (35 cycles at 96°C for 30 s, 58°C for 15 s and 60°C for 4 min). Each mutation was verified by sequencing in both directions. Mutations were further confirmed by restriction analysis (Table 2). After PCR, the purified DNA was digested with the appropriate enzyme (New England Biolabs, Schwalbach/Taunus, Germany) according to the manufacturer's specifications and separated on agarose gels (FMC BioProducts, Rockland, Me.).

Raster3D picture of rabbit GPI at 2.5-Å resolution

The figure is based on X-ray structures of pig (Shaw and Muirhead 1977; Davies 1991) and rabbit GPI (Davies and Muirhead, unpub-



**Fig. 2** Raster3D picture of the X-ray structure of rabbit glucose-6-phosphate isomerase (GPI). The structure of rabbit GPI solved to 2.5-Å resolution (Davies and Muirhead, unpublished) showing those positions in the dimer corresponding to the mutations in human GPI. Also shown are residues located near to the active site. For clarity active-site residues and mutations are shown for one monomer only, except for His389, which is on the second monomer. One monomer is coloured yellow, the other, blue. Active-site residues are green; mutated residues identified in this paper are red and labelled (numbering as in human GPI). Image produced using MOLSCRIPT (Kraulis 1991) and RASTER3D (Merritt and Murphy 1994)

lished). It was produced using the programs Molscript (Kraulis 1991) and Raster3D (Merritt and Murphy 1994).

## Results

Earlier studies in our laboratory had identified two patients with the variant enzymes GPI Homburg and GPI Calden, respectively, at the haematological and biochemical level (Schröter et al. 1985; Eber et al. 1986; Neubauer et al. 1990). Those patients (I, II/1, II/2) with severe GPI deficiency showed the characteristic haemolytic anaemia (Table 1) and a significant decrease in granulocyte function. In addition, the patient with GPI Homburg showed neurological impairment, e.g. myopathy and mental retardation (Schröter et al. 1985).

In order to analyse the underlying molecular defect we determined the nucleotide sequence of all the exons and intron-exon junctions of the *GPI* gene on PCR-amplified DNA from patient I (GPI Homburg) and the two siblings, II/1, II/2, (GPI Calden) together with their families. Four different missense mutations were detected by sequencing and then confirmed by restriction analysis (Tables 1, 2; Fig. 1). Patient I was shown to be compound heterozygous for mutations located in exon 1 (H20P) and in exon 12

(L339P), respectively. The patient's mother is heterozygous for the exon 12 mutation; his father was not available for molecular analysis. In the second family studied, the two affected siblings (II/1, II/2) are compound heterozygous for substitutions in exon 13 (H389R) and in exon 18 (L517V), respectively. Their parents are heterozygous for the nucleotide changes in exon 13 (mother) and exon 18 (father), respectively (Table 1). To exclude the possibility that these novel mutations are silent polymorphisms, identical genomic fragments were amplified from 60 unaffected individuals of European origin and examined for the presence of these four base substitutions. The base substitutions detected in the GPI-deficient patients I, II/1 and II/2 were not found in any of these DNA samples, thus making the existence of polymorphism unlikely. An analysis of two known polymorphisms in the GPI gene (Xu and Beutler 1994) revealed that the mutations identified here are in linkage with nucleotides 489A and 1356G, respectively.

To establish a structure-function relationship between the identified mutations and the phenotypes of the patients, we analysed the location of the affected amino acids. The three-dimensional structure of the GPI enzyme is based on X-ray data of pig (Shaw and Muirhead 1977; Davies 1991) and rabbit GPI (Davies and Muirhead, unpublished). It is assumed that the structure in human will be homologous. The crystal structure of pig GPI at medium resolution (Davies 1991) indicates that His20 is the last residue in an  $\alpha$ -helix and lies on the surface of the dimer. Leu339 is in an internal  $\beta$ -strand and immediately precedes a proline residue. Neither of these residues is close to the active site (Fig. 2), but the insertion of proline in elements of regular secondary structure would be expected to affect the correct folding and hence both the structure and the activity of the enzyme. Therefore there is little doubt of the causative role of the exon 1 and 12 mutations in the determination of the severe phenotype in patient I. He exhibits a drastically decreased GPI activity in all tissues examined (Schröter et al. 1985; Table 1), i.e. erythrocytes (7% of normal), leukocytes (9%), skeletal muscle (17%) and cerebrospinal fluid (0%). Both mutations found in the second family are in highly conserved regions of the molecule. His389 is a proposed active-site residue. Leu517 is close to the essential Lys519 (Fig. 2). They are both at the subunit interface and lie in parts of the structure that undergo conformational changes when a competitive inhibitor is bound (Shaw and Muirhead 1976). It is probable that the molecule would fold correctly, but residues in the active site are mutated and the correct conformational change on binding substrate may be inhibited. This suggests that both mutations would affect the catalytic activity of the enzyme and be responsible for the decreased GPI activity in both patients (Table 1).

## Discussion

Given these results (Table 1) we were able to compare the haematological and biochemical data with the modifications of the primary structure of the enzyme. As an effect of

the reduced GPI activity, increased amounts of the substrate, glucose-6-phosphate, could be measured (Table 1; Schröter et al. 1985; Eber et al. 1986; Neubauer et al. 1990). The fourfold elevated glucose-6-phosphate concentration in patient I is probably indicative of a serious perturbation of glycolysis. We further show that both GPI variants exhibit enzyme instability (Table 1), a characteristic that has been related to the loss of enzyme activity. The  $K_m$  value for fructose-6-phosphate is slightly reduced in the two siblings but threefold lowered in patient I, implying a higher affinity of the mutated enzyme for its substrate (Eber et al. 1986; Neubauer et al. 1990). This in turn would impair the balance between binding and dissociation of the substrate, thus resulting in a reduced turnover.

Like GPI from other species (Shaw and Muirhead 1977), the human GPI that is under current investigation is composed of two identical subunits of  $M_r$  66,000. Both subunits contain a large and a small domain, each having a parallel  $\beta$ -sheet core surrounded by  $\alpha$ -helices linking the  $\beta$ -strands, a structure typical of glycolytic enzymes (Achari et al. 1981). The active site is situated in a cleft between the large and small domains of the monomer, close to the subunit interface, and is formed by the association of the two subunits (Shaw and Muirhead 1976). Enzyme deficiency may result from instability of the protein due to alterations either in the individual monomer or in the subunit interface. Due to the insertion of proline this could be a consequence of the mutations H20P and L339P (Fig. 2). On the other hand, structural alterations affecting the active site and/or the subunit interface have been taken to suggest alterations in specific activity and other kinetic properties. This seems to be the case in the H389R and L517V mutations as both of them are located in the active site and at the subunit interface (Fig. 2).

It is worthwhile considering the possible pathophysiological significance of the relationship between the neurological symptomatology and GPI deficiency. The involvement of GPI/NLK in neurological processes is exemplified by the finding that NLK has been associated with motor neurone disease (Gurney et al. 1984) and with the neuropathology sometimes found in patients with AIDS (Lee et al. 1987). In a bioassay it was shown that the dimer is responsible for catalytic activity (GPI) and the monomer for neurotrophic (NLK) activity (Mizrachi 1989). Besides our patient I (GPI Homburg), neurological symptoms have also been found in patients with the variants GPI Utrecht (Hellerman and van Biervliet 1975), GPI Paris (Kahn et al. 1978), GPI Mount Scopus (Shalev et al. 1993), and with a GPI variant described by Zanella et al. (1980). Until now, only GPI Mount Scopus has been characterized at the molecular level (Beutler et al. 1997). In view of a possible effect of GPI mutations on the central nervous system through an effect on NLK activity, it is tempting to set up the following, somewhat speculative, hypothesis: mutations that specifically destabilise the monomer seem to destroy both catalytic (GPI) and neurotrophic (NLK) activity. This could explain the neurological symptoms of patient I. Mutations at the active site that are in or close to the subunit interface and destabilise the dimer, but not necessarily the monomer,

lead to protein with a loss of catalytic activity but intact neurotrophic properties. As a result the clinical picture of the two siblings (II/1, II/2) is characterized by chronic haemolytic anaemia alone without any neurological deficits.

Ten out of 19 GPI missense mutations identified so far (reviewed in Beutler et al. 1997) support our hypothesis as they occur in or close to the subunit interface (T195I, T224M, R347C, R347H, T375R, L487F, E495K, I525T and D539N; R273H is a possible active-site residue). The patients carrying these mutations show no neurological deficit. Similarly, the patients harbouring the other GPI mutations exhibit no neurological symptoms. In the case of GPI Mount Scopus (R347C) it was impossible to ascertain whether the sensineural deficits of the patient were directly related to the enzyme deficiency, or were secondary to neonatal hyperbilirubinaemia and tissue hypoxia due to profound anaemia (Shalev et al. 1993). In contrast, our investigations revealed that the neuromuscular symptoms of patient I are directly related to enzyme deficiency (Schröter et al. 1985). However, in a randomly associating system, the two different mutated GPI monomers (A\*, A°) of a compound heterozygous patient would provide A\*A\*, A\*A° and A°A° dimers. Unfortunately, neither the ratios amongst the two homodimers and the heterodimer are known, nor which species is most abnormal or unstable. Thus, in order to determine the pathophysiology of each of these mutants unambiguously it is necessary to find homozygotes or produce them by genetic engineering.

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