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ORIGINAL INVESTIGATION

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Identification of missense, nonsense, and deletion mutations in the *GRHPR* gene in patients with primary hyperoxaluria type II (PH2)

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Abstract Primary hyperoxaluria type II (PH2) is a rare disease characterized by the absence of an enzyme with glyoxylate reductase, hydroxypyruvate reductase, and D-glycerate dehydrogenase activities. The gene encoding this enzyme (*GRHPR*) has been characterized, and a single mutation has been detected in four PH2 patients. In this report, we have identified five novel mutations. One nonsense mutation (C295T) results in a premature stop codon at codon 99. A 4-bp deletion mutation has been found in the 5' consensus splice site of intron D, resulting in a predicted splicing error. Three missense mutations have been detected, including a missense transversion (T965G) in exon 9 (Met322Arg), a missense transition (G494A) in the putative co-factor binding site in exon 6 (Gly165Asp), and a substitution of an adenosine for a guanine in the 3' splice site of intron G. The functional consequences of the missense transversion and transition mutations have been investigated by transfection of cDNA encoding the mutated protein into COS cells. Cells transfected with either mutant construct have no enzymatic activity, a finding that is not significantly different from the control (empty) vector ($P<0.05$). These results further confirm that mutations in the *GRHPR* gene form the genetic basis of PH2. Ten of the 11 patients that we have genotyped are homozygous for one of the six mutations identified to date. Because of this high proportion of homozygotes, we have used microsatellite markers in close linkage with the *GRHPR* gene to investigate the possibility that the patients are the offspring of related individuals. Our data suggest that two thirds of our patients are the offspring of either closely or distantly related persons. Furthermore, genotyping has revealed the possible presence of a founder effect for the two most common mutations and the location of the gene near the marker D9S1874.

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Introduction

Primary hyperoxaluria type II (PH2) is a rare monogenic disease with an autosomal recessive pattern of inheritance. It results from the absence of an enzyme with D-glycerate dehydrogenase (DGDH) activity (Williams and Smith 1968). The enzyme also has glyoxylate reductase (GR) and hydroxypyruvate reductase (HPR) activities, which are thought to be the favored reactions in mammals (Van Schaftingen et al. 1989). The gene coding for this enzyme has been assigned the locus designation GRHPR by the human genome nomenclature committee (http://www.gene.ucl.ac.uk/nomenclature/). PH2 is characterized by an elevated urinary excretion of oxalate and L-glycerate (Williams and Smith 1968; Chlebeck et al. 1994), caused by the absence of GR and HPR activities, respectively. Increased plasma oxalate in PH2 patients can cause nephrolithiasis and nephrocalcinosis and can, in some cases, result in renal failure and systemic oxalate deposition. PH1, characterized by increased urinary oxalate and glycolate excretion, results from a deficiency of the liver-specific peroxisomal enzyme alanine-glyoxylate aminotransferase. Historically, the prognosis for PH2 patients has been more favorable and the disease less common than PH1. Whereas PH2 is still thought to have a more favorable prognosis than PH1 (Milliner et al. 1998), the severity of the clinical course and prevalence of PH2 have recently been brought into question. End-stage renal disease has now been documented in three of the 24 patients reported in the literature (Kemper et al. 1997). In addition, PH2 may be more common than was previously suspected, most probably because of the lack of availability of assays for urinary glycolate and L-glycerate. Implementation of these discriminatory assays at the Mayo Clinic has led to

the recognition of PH2 in 20% of patients previously diagnosed with PH1 (Chlebeck et al. 1994).

GRHPR is a member of a family of NAD/NADP-dependent dehydrogenases. Although the crystal structure of the human enzyme has not been determined, crystallographic data is available for the enzyme from *Hyphomicrobium methylovorum* (Goldberg et al. 1994). The high degree of similarity between the GRHPR protein sequences in human and other organisms, including *H. methylovorum* (Cramer et al. 1999), allows the use of the enzyme of the latter as a tentative three-dimensional model for the human protein. Goldberg et al. (1994) have concluded that the enzyme forms a symmetrical dimer, with each monomer consisting of two structurally similar domains, identified as the co-factor binding domain and the catalytic domain. The domains are separated by a deep cleft believed to form the putative binding sites for the co-factor and substrate. The amino acids important in these sites are highly conserved among the GRHPR protein sequences from eubacteria, plants, and animals and among the predicted protein sequences from archaea and fungi (Cramer et al. 1999). This structural information may be useful in elucidating the functional characteristics of the enzyme in normal and PH2 individuals.

The gene that is mutated in PH2 patients has only recently been characterized. We have described a cDNA that encodes DGDH activity (Cramer et al. 1998) and have subsequently demonstrated that it also encodes HPR and GR activities (Cramer et al. 1999). This cDNA has been identified as an 1198-nucleotide clone encoding a 984-nucleotide open reading frame. The open reading frame translates to a predicted 328-amino-acid protein with a predicted mass of 35,563 Da. Another group has identified an identical cDNA with HPR and GR activities, encoding a 328-amino-acid protein (Rumsby and Cregeen 1999). We have recently determined the genomic structure of the GRHPR gene and demonstrated that it contains nine exons and eight introns spanning approximately 9 kb pericentromeric on chromosome 9, within the reference interval D9S1874–D9S273 (Cramer et al. 1999). A schematic diagram of the genomic structure and intron/exon nomenclature used in this report is given in Fig. 1. Polymerase chain reaction/single-stranded conformation polymorphism (PCR-SSCP) analysis and sequence analysis of four PH2 patients (representing two pairs of siblings) have revealed a single nucleotide deletion in exon 2 resulting in a frame-shift mutation. The mutated gene is predicted to encode a truncated protein of 44 amino acids, which lacks the putative co-factor and substrate binding sites; this is believed to be the disease-causing mutation for these patients (Cramer et al. 1999).

In this report, we describe our analysis of mutations in the *GRHPR* gene from seven additional PH2 patients. Five new mutations have been identified that include missense, nonsense, and deletion mutations. We describe our results concerning our functional analysis of the missense mutations. Because of the high proportion of homozygous mutations observed, we have also investigated the possibility that the patients are the offspring of related individuals and comment on the presence of a founder effect for the most common mutations.

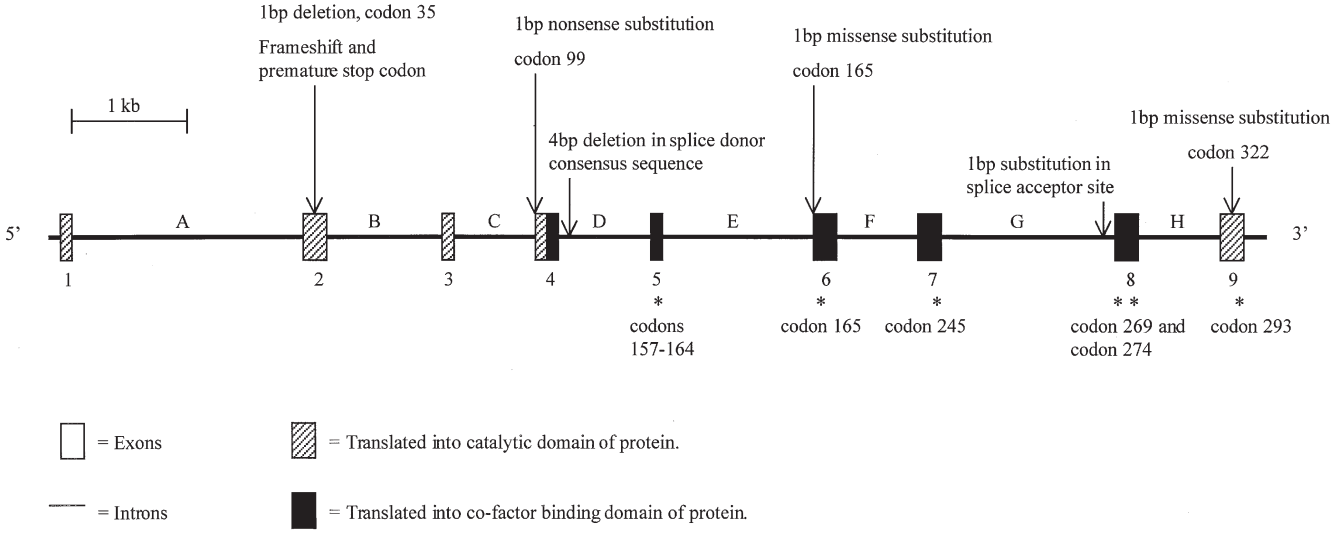


Fig. 1 Distribution of the six known mutations in the human GRHPR gene. *Boxes* Exons, *lines* introns, *shaded boxes* sequence translated into the catalytic domain of the protein, *solid boxes* sequence translated into the co-enzyme binding domain of the protein. Conserved sequences important in co-factor and substrate binding are designated by an *asterisk* (based on crystallographic data for the enzyme from *Hyphomicrobium methylovorum*; Goldberg et al. 1994)

Materials and methods

Patients

All new patients studied in this report were confirmed as being PH2 patients by the definitive diagnostic urinary oxalate and urinary L-glycerate assays. DNA samples from four patients were obtained from Ospedale Mauriziano Umberto in Torino, Italy. Three

patients were of Caucasian Italian (CIt) descent, while the fourth was of Indian Asian (InAs) descent. Both CIt1 and CIt2, a 50-year-old male and 26-year-old female, respectively, are currently on dialysis. Both CIt3 and InAs1, a 4-year-old male and 8-year-old female, respectively, have a normal glomerular filtration rate and nephrolithiasis. Patients CIt1 and InAs1 have previously been reported in literature (Marangella et al. 1995). Blood samples were obtained from two patients in the USA, a Caucasian American (CA1) male and an African American (AA1) adolescent female. AA1 is currently on dialysis after a failed renal transplant. Lastly, a blood sample of an 8-year-old female (GER1) was obtained from the University Children's Hospital, Hamburg, Germany. This patient has been previously reported in the literature (Kemper and Müller-Wiefel 1996). All protocols were approved by the Institutional Review Board of Wake Forest University School of Medicine.

PCR-SSCP technique

Blood was collected from the North American and German patients and shipped on ice by courier to Wake Forest University School of Medicine. Total genomic DNA was isolated by using the QIAmp DNA Blood Mini Kit (Qiagen, Valencia, Calif.). For the Italian patients, blood was collected, and total genomic DNA was isolated at the Renal Stone Laboratory, Ospedale Mauriziano Umberto, Italy. PCR-SSCP was applied to all nine exons of the PH2 patients by using a method previously described (Cramer et al. 1999). Any exons that displayed aberrant SSCP patterns compared with the wild-type control were re-amplified with non-radioactive primers and sequenced with an ABI Prism 377 DNA Sequencer at the Nucleic Acid Sequencing Facility of the Comprehensive Cancer Center of Wake Forest University (CCCWFU).

Site directed mutagenesis – construction of missense mutants

Site-directed mutagenesis was used to construct mutant clones to represent the missense mutations of patients AA1 and InAs1. The cDNA for human GRHPR was previously subcloned into the mammalian expression vector pcDNA3.1zeo(–) (Invitrogen, Carlsbed, Calif.) at the *Xba*I and *Kpn*I restriction sites (Promega, Madison, Wis.; pGRHPR3.1; Cramer et al. 1999).

Site-directed mutagenesis by PCR was used to modify the pGRHPR3.1 sequence (Higuchi et al. 1988). By this method, two overlapping fragments of the target sequence were PCR-amplified. Each reaction involved the use of one flanking primer that hybridized to the vector sequence, just outside either the *Xba*I or *Kpn*I restriction sites, and one internal primer that hybridized at the site of the mutation and contained the mismatched base. Internal primer sequences for AA1 were: 5'-GGGGAGCCGA**G**GCCTA-GTGAACTCAA-3' and 5'-TTCACTAGGC**C**TCGGCTCCCCT-CTCA-3'; and for InAs1 were: 5'-GGCGCATAG**A**CCAGGCC-ATTGCTCG-3' and 5'-ATGGCCTGG**T**CTATGCGCCCCAGC-3'. Flanking primer sequences were: 5'-CTCACTATAGGGAGACC-CAAGCTGGC-3' and 5'-GGTCAAGGAAGGCACGGGGGA-GG-3'. The modifications were a thymine to a guanine (methionine to arginine, codon 322) and a guanine to an adenosine (glycine to aspartate, codon 165) for patient AA1 and InAs1, respectively. Each PCR was performed with 100 ng pGRHPR3.1, 15 pmol each primer, 0.64 mM dNTPs, 1×ThermoPol Reaction Buffer (containing 2 mM $MgCl_2$; New England Biolabs, Beverly, Mass.), in a final volume of 100 µl. Amplifications consisted of 94°C for 5 min, 80°C for 5 min (2 U $Vent_R$ DNA polymerase, supplied by New England Biolabs, was added at this stage), followed by 30 cycles of 94°C for 45 s, 63°C for 1 min, and 72°C for 1 min, and a final extension (72°C) of 7 min. The PCR products were separated on a 1.5% agarose gel, and the appropriate band was excised and purified by using the Geneclean II Kit (Bio 101, Vista, Calif.). The two overlapping fragments were combined in a second round of PCR amplification with the flanking primers. The PCR mix was identical to that above, except 50 ng of each DNA fragment was used. Amplification consisted of 94°C for 5 min, 80°C for 5 min (2 U $Vent_R$ DNA polymerase was added at this stage), followed by 30 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min 30 s, with a final extension (72°C) of 7 min. The resulting mutated DNA fragment was gel-purified (Geneclean II Kit) and subcloned back into pcDNA3.1zeo(–) at the *Hind*III and *Nhe*I restriction sites to create plasmids pmGRHPR3.1 (InAs1 and AA1). Plasmid DNA was purified by using the Wizard Plasmid kit (Promega). The correct introduction of the mutant base and the absence of polymerase-introduced sequence errors was confirmed by automated fluorescent dideoxy nucleotide sequence analysis of overlapping 500-bp fragments, as described above for exon PCR products.

Transient transfection and enzymatic activities

COS cells were obtained from the Tissue Culture Core Laboratory of CCCWFU. Transfection experiments were performed by using Lipofectamine (Gibco BRL, Grand Island, N.Y.) as previously described (Cramer et al. 1999). A mass of 3.6 µg of each plasmid, viz., vector alone [pcDNA3.1zeo(–)], wild type GRHPR (pGRHPR3.1), or the missense mutants (pmGRHPR3.1 AA1 and InAs1), was co-transfected with 1.2 µg luciferase reporter plasmid (pGL3 control; Promega) to control for differences in transfection efficiencies. Each co-transfection was performed in triplicate. Some 23 h after transfection, the cells were scraped off the plates, spun briefly, and stored as a cell pellet at –70°C until required.

The cells were lysed by sonication in 100 µl 100 mM TRIS, pH 8.5. Protein concentration was determined by the BCA microassay according to the manufacturer's protocol (Pierce, Rockford, Ill.). Luciferase activity was measured in 20-µl aliquots of cell lysate at 37°C, by using the Promega luciferase assay system, in a Turner TD-20e luminometer according to the manufacturer's protocol.

DGDH, HPR, and GR enzymatic activities were determined as previously described (Cramer et al. 1999). DGDH activity is expressed as V (pmol hydroxypyruvate phenylhydrazone formed/min per milligram protein per light unit). HPR and GR assays were performed in the presence of 100 µM NADPH. The assay was initiated by the addition of enzyme, and the A_{340} recorded every 15 s, over a period of 180 s, by using a Beckman DU-640 spectrophotometer. The initial linear rate was used to determine enzymatic activity in each extract.

Genotyping by microsatellite markers

A total of 11 patients, seven from this report and four studied previously (Cramer et al. 1999), and 93 controls were typed by using three microsatellite markers: D9S1874, D9S1787, and D9S273. Radiation hybrid analysis performed by the Sanger Center (http://www.sanger.ac.uk/) mapped the gene within the reference interval D9S1874–D9S273. This interval is 5 cM in length, with D9S1787 being located 1.1 cM centromeric to D9S273. The polymorphic regions containing dinucleotide repeats were PCR-amplified with end-labeled oligonucleotide primers. Genethon primer sequences (Evry, France) were used for each of these markers (Weissenbach 1996). End-labeling of the forward primer was performed in a reaction containing 10 µM primer, 2.5 pmol γ^{32}P-ATP at 3000 Ci/mmol, 1× kinase reaction buffer, and 3 U T4 polynucleotide kinase (Promega), incubated at 37°C for 30 min. Each PCR was performed by using 50–100 ng genomic DNA, 1 µM ^{32}P end-labeled forward primer, 1 µM unlabeled reverse primer, 0.2 µM dNTPs, 1× *Taq* DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, and 0.3 U *Taq* DNA polymerase (Promega), in a final volume of 10 µl. Amplifications consisted of 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, with a final extension (72°C) of 7 min. An aliquot of 1 µl of each reaction was mixed with 7 µl sequencing stop buffer (87% formamide, 0.02 M EDTA pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and 12 µl H_2O, denatured at 95°C for 3 min, and snap-cooled on ice. An aliqot of 2 µl of each cooled sample was loaded on a 6% polyacrylamide/42% urea sequencing gel and run at 70 W for 3–5 h. Kodak X-OMAT film was exposed to gels overnight at –70°C and developed in a Kodak M35A X-OMAT processor. In scoring,

the samples producing the band with the slowest mobility were arbitrarily designated as allele 1. Haplotypes for the patients were constructed with reference to marker order, in a family context. For probands whose family was not available, haplotypes were constructed by using homozygosity. Allele frequencies for the DNA markers were determined from 186 control alleles. The control group consisted of 38 African American, 52 Caucasian American, and three Caucasian Italian individuals.

Statistics

Student's *t*-test was applied ($P<0.05$) to the raw data from the enzyme assay in order to determine whether the mean enzymatic activities of each clone were significantly different. Statistical comparison of allele frequencies between PH2 patients and the control group was performed by using the Fexact program (Exact Inference for Unordered R×C Contingency Tables; Division of Biostatistics and Epidemiology, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, Mass.; Mehta and Patel 1983). *P* values were calculated by using the Monte Carlo Algorithm.

Results

Identification of new mutations and their functional consequences

In a previous report, we identified a single nucleotide deletion in exon 2 of the *GRHPR* gene in four PH2 patients from two unrelated families (two pairs of siblings; Cramer et al. 1999). This deletion results in a frame-shift and introduction of a premature stop codon at codon 44. These data supported the hypothesis that mutations in the *GRHPR* gene result in PH2. To test this hypothesis further, we evaluated the *GRHPR* gene of seven additional PH2 patients for deviations from the wild-type sequence. PCR-SSCP assays, previously described by us (Cramer et al. 1999), were used to screen each of the nine exons, including intron-exon boundaries, for aberrant electrophoretic mobility. Exons displaying abnormal SSCP patterns were subsequently sequenced. Two patients, CA1 and GER1 (see above for patient descriptions and nomenclature), were found to be homozygous for the known deletion mutation in exon 2 (data not shown). Among the remaining five patients, five novel mutations were discovered and are discussed below.

Nonsense mutation

Figure 2A shows the PCR-SSCP analysis of exon 4 of patients CIt1 and CIt2. The double-headed arrow points to the abnormal migration of the PCR product for the two patients in lanes 3 and 8, compared with the wild-type control in lane 1. Figure 3A shows the sequencing results from exon 4 of patient CIt1. The results reveal a single nucleotide substitution: a thymine for a cytosine in codon 99. The sequencing results for patient CIt2 were identical (data not shown). This substitution creates an amino acid change from an arginine (CGA) to a stop codon (TGA). The predicted result is a truncated protein of 98 amino acids, instead of 328, lacking the putative co-factor and substrate binding sites. Both patients are homozygous for this mutation (Fig. 3A). Furthermore, the PCR-SSCP pattern (Fig. 2A, lanes 9 and 10) and subsequent sequencing (not shown) confirm the parents of patient CIt2 to be heterozygotes. This mutation is predicted to cause the loss of a *Bam*HI restriction enzyme site (Table 1).

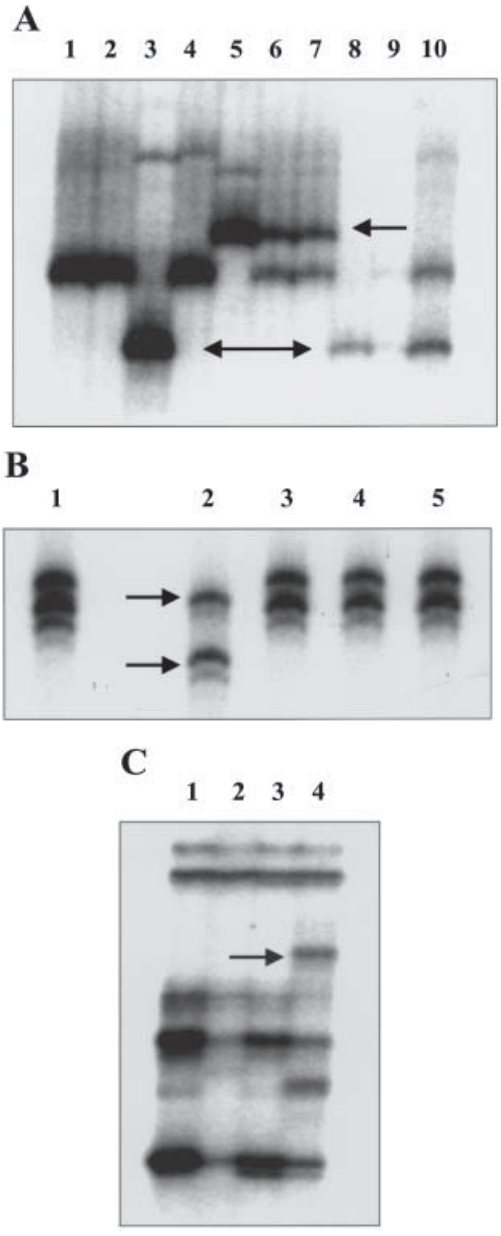
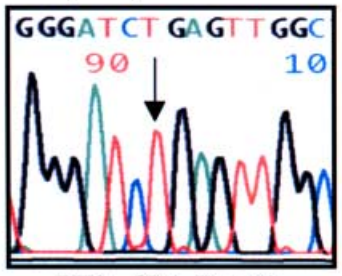
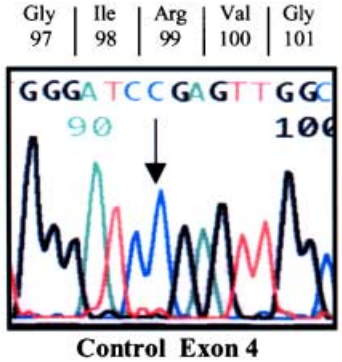


Fig. 2A–C Autoradiograms of PCR-SSCP analysis of exons 4, 6, and 9 of *GRHPR* for patients compared with normal controls. *Arrows* Shift in band pattern in PH2 patients. **A** Autoradiogram of exon 4. *Lane 1* Normal control, *lanes 2, 4* PH2 patients with normal exon 4, *lane 3* patient CIt1, *lane 5* patient CIt3, *lanes 6, 7* parents of CIt3, *lane 8* patient CIt2, *lanes 9, 10* parents of CIt2. **B** Autoradiogram of exon 9. *Lanes 1, 4, 5* Normal controls, *lane 2* patient AA1, *lane 3* PH2 patient with normal exon 9. **C** Autoradiogram of exon 6. *Lane 1* Normal control, *lanes 2, 3* PH2 patients with normal exon 6, *lane 4* patient InAs1

A

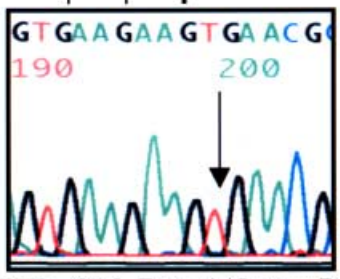


PH2 - CIt1 Exon 4

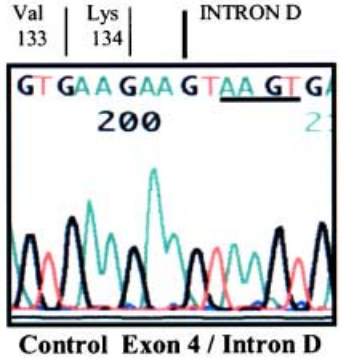


Control Exon 4

B

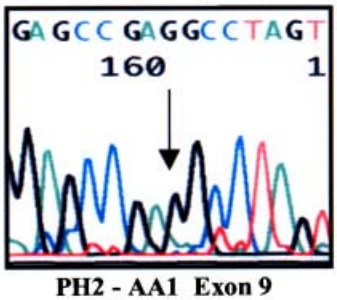


PH2 - CIt3 Exon 4 / Intron D

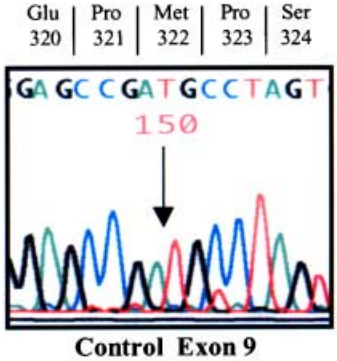


Control Exon 4 / Intron D

C

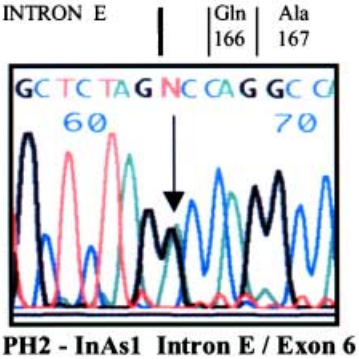


PH2 - AA1 Exon 9

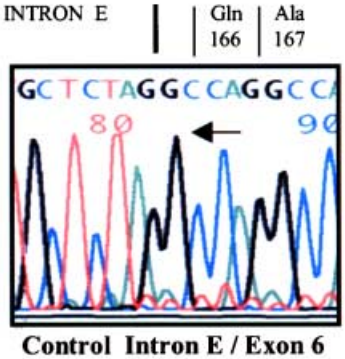


Control Exon 9

D

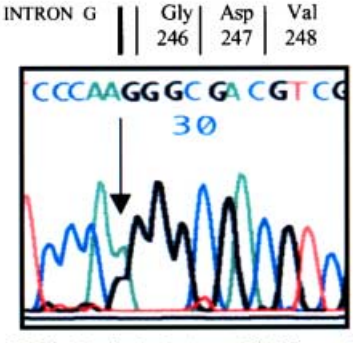


PH2 - InAs1 Intron E / Exon 6

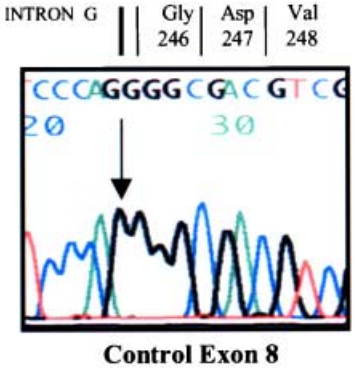


Control Intron E / Exon 6

E



PH2 - InAs1 Intron G / Exon 8



Control Exon 8

Table 1 Characteristics of the six known mutations in the *GRHPR* gene of PH2 patients (– loss of a restriction site in the presence of the mutated base, + addition of a restriction site in the presence of the mutated base, n/a: not applicable)

Exon/intron	Nucleotide[a]	cDNA change	Amino acid change	Frequency in 18 PH2 alleles	Affected restriction site
Exon 2	103delG	1 bp deletion	Asp35Thr frameshift aa 44/stop	8 (44.4%)	None
Exon 4	C295T	Nonsense transition	Arg99stop	4 (22.2%)	–*Bam*HI
Exon 9	T965G	Missense transversion	Met322Arg	2 (11.1%)	+*Stu*I
Intron D	del 5' splice consensus site AAGT	4 bp deletion and splicing error	n/a	2 (11.1%)	None
Exon 6	G494A	Missence transition	Gly165Asp	1 (5.6%)	+*Xba*I
Intron G	G 3'splice site A	Missence transition and splicing error	n/a	1 (5.6%)	None

[a]Nucleotides are numbered in reference to the first nucleotide of the initiation codon

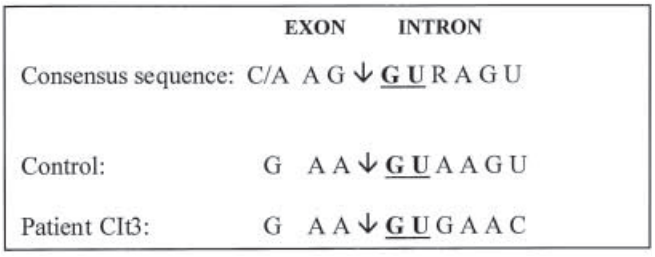


Fig. 4 The four-base deletion in patient CIt3 disrupts the 5' splice site consensus sequence required for correct spliceosome binding. The nucleotide sequence at the boundary of exon 4 and intron D of patient CIt3 and a normal control are compared with the consensus sequence required for RNA splicing in higher eukaryotes (Blencowe 2000). *Arrows* Exon/intron boundary, *G* guanine, *A* adenosine, *C* cytosine, *U* uracil, *R* purine

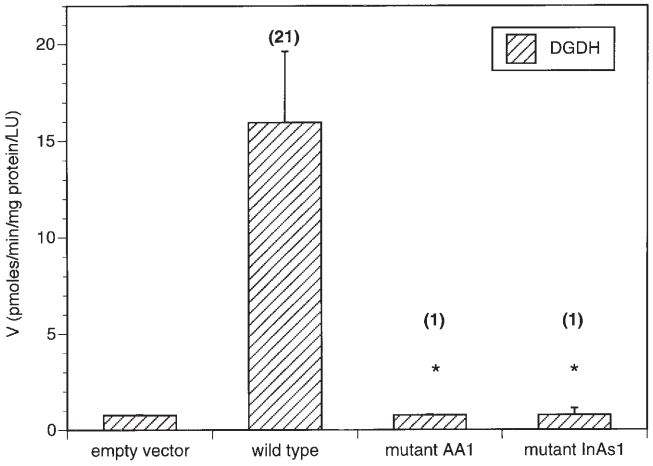


Fig. 5 The missense mutations in patients AA1 and InAs1do not encode a functional GRHPR gene. COS 1 cells were co-transfected with luciferase reporter vector and empty vector (pcDNA3.1), or wild type (pGRHPR3.1), or mutant AA1 (pmGRHPR3.1 AA1), or mutant InAs1 (pmGRHPR3.1 InAs1). Enzyme activities were determined as described. *DGDH* D-glycerate dehydrogenase activity, *bars* mean ± SEM (n=3), *numbers in parentheses* fold-increase in enzyme activity relative to the empty vector, *asterisk* significantly different from the corresponding wild-type control vector and not significantly different from the corresponding empty vector (P<0.05).

Deletion mutation

PCR-SSCP analysis of exon 4 of patient CIt3 is also shown in Fig. 2A. The single-headed arrow points to the abnormal migration of the PCR product for CIt3 in lane 5, compared with the wild-type control in lane 1. Sequencing results reveal a 4-bp deletion immediately downstream of the 5' splicing site (donor site) in intron D (Fig. 3B). The patient is homozygous for this mutation, whereas the PCR-SSCP pattern (lanes 6, 7) and subsequent sequencing (not shown) have revealed the parents to be heterozygous. Figure 4 shows the consensus sequence required for the proper functioning of the spliceosome at the 5' splice site (Blencowe 2000) compared with the sequences found in a normal control and patient CIt3. Presumably, the lack of consensus sequence would prevent the spliceosome from binding to the 5' splice site, thereby preventing the removal of intron D.

◀**Fig. 3A–E** Nucleotide sequence analysis of mutated exons compared with normal control. An isolated section of each electropherogram is shown. The deduced nucleotide sequence and the codons corresponding to the hGRHPR protein are located *above* each panel. *Arrows* Affected nucleotide(s). **A** Electropherogram of exon 4 of patient CIt1 (same as CIt2, not shown), compared with normal control. **B** Electropherogram of exon 4 and intron D of patient CIt3, compared with normal control. **C** Electropherogram of exon 9 of patient AA1, compared with normal control. **D** Electropherogram of exon 6 of patient InAs1, compared with normal control. **E** Electropherogram of intron G and exon 8 of patient InAs1, compared with normal control

Missense mutations

PCR-SSCP analysis of exon 9 of patient AA1 demonstrated the presence of an aberrant product when compared with the wild-type control (Fig. 2B, lane 2). Sequencing results revealed a single nucleotide substitution: a guanine for a thymine in codon 322 (Fig. 3C), which is predicted to introduce a *Stu*I restriction enzyme site. The patient is homozygous for this mutation. This creates an

amino acid change from a methionine (ATG) to an arginine (AGG). This site has been shown to be either conserved or subject to conservative substitution in the predicted GRHPR proteins from various organisms (Cramer et al. 1999). The effect of this nucleotide substitution on enzyme activity was investigated by construction of a mutant clone and subsequent transfection into COS cells, as detailed above. A graphical comparison of DGDH enzyme activity between the control (empty vector), the wild type (pGRHPR3.1), and the mutant (pmGRHPRAA1) clone is shown in Fig. 5. This figure demonstrates that the enzymatic activity of the mutant clone is significantly less than that of the wild-type control and not significantly different from the background control. Furthermore, the results of the GR and HPR assays followed the same pattern (data not shown).

PCR-SSCP analysis was also completed for all exons of patient InAs1. The only exon to demonstrate an aberrant SSCP product when compared with the wild-type control was exon 6 (Fig. 2C, lane 4). Sequencing results revealed the patient to be heterozygous for a single nucleotide substitution: an adenosine for a guanine in codon 165 (Fig. 3D), which is predicted to introduce a *Xba*I restriction enzyme site. This creates an amino acid change from a glycine (GGC) to an aspartate (GAC). This amino acid is 100% conserved in the GRHPR from species representing Archaebacteria, Eubacteria, plants, fungi, and animals and is located within the putative co-factor binding site (Cramer et al. 1999). The alteration of this highly conserved residue was predicted to affect protein function adversely. This prediction was investigated by construction of a mutant clone and subsequent transfection into COS cells. Figure 5 shows that the DGDH enzymatic activity of pmGRHPR InAs1 is significantly less than that of the wild-type control (pGRHPR3.1) and not significantly different from the background control (pcDNA3.1). Likewise, the GR and HPR enzymatic activities for pmGRHPR InAs1 are significantly less than the wild type and not significantly different from the empty vector (data not shown).

No other exons had abnormal SSCP patterns for patient InAs1. However, sequencing of all exons and intron boundaries revealed a heterozygous substitution of an adenosine for a guanine in the 3' splice site (acceptor site) of intron G (Fig. 3E), presumably on the opposite chromosome to the exon 6 mutation. The absence of a correct acceptor site is predicted to cause aberrant splicing between exons 7 and 9, with all or part of exon 8 being spliced out of the mRNA and presumably resulting in an unstable product that would be quickly degraded. Patient InAs1 is the only compound heterozygote for a *GRHPR* mutation that we have identified.

Summary of mutations

Table 1 contains a summary of the six known *GRHPR* mutations, their position, and consequences for the nucleotide and amino acid sequence. Also tabulated is the frequency of each mutation among the alleles studied by our group. Only 18 alleles were included in this analysis (the alleles of siblings were not counted twice), which demonstrated the exon 2 deletion as the most common, being present in just under half the alleles. The affected restriction site (if any) is also noted, because of the potential use for genetic screening. Figure 1 gives a diagrammatical representation of the gene and the known mutations. The position of mutations is given relative to the catalytic and co-factor binding domains of the protein, in addition to the specific residues highly conserved in the putative substrate and co-factor binding sites of the *H. methylovorum* protein (Goldberg et al. 1994). In summary, the mutations in exon 2, exon 4, and intron D would result in truncated proteins lacking all the putative co-factor and substrate binding sites, whereas the substitution in

Table 2 Haplotype analysis of PH2 patients and available parents, as generated by three microsatellite markers(*nd* not determined or could not be amplified for indeterminate reasons, id indeterminable)

Mutation	Patient/Parent	Allelotypes			Haplotypes	
		D9S1874	D9S1787	D9S273		
Exon 2	GER1	5,5	9,9	8,8	5,9,8	5,9,8
	CA1	5,5	5,10	5,6	id	id
	CA2[a]	5,5	10,10	8,8	5,10,8	5,10,8
	CA3[a]	5,5	10,10	8,8	5,10,8	5,10,8
	CA4[b]	5,5	5,5	8,8	5,5,8	5,5,8
	CA5[b]	5,5	5,5	8,8	5,5,8	5,5,8
Exon 9	AA1	4,4	3,3	7,7	4,3,7	4,3,7
Exon 6/Intron G	InAs1	nd	4,9	8,9	id	id
Exon 4	CIt1	4,4	6,6	8,8	4,6,8	4,6,8
	CIt2	4,4	6,9	4,6	4,6,6	4,9,4
	Father –CIt2	4,6	6,6	6,6	4,6,6	6,6,6
Intron D	CIt3	4,4	5,9	8,8	4,5,8	4,9,8
	Mother – CIt3	4,7	5,6	8,8	4,5,8	7,6,8
	Father – CIt3	4,4	9,9	7,8	4,9,8	4,9,7

[a]Siblings previously reported in literature (Cramer et al. 1999)
[b]Siblings previously reported in literature (Cramer et al. 1999)

Table 3 Comparison of allele frequencies between PH2 patients and non-PH2 controls

D9S1874			D9S1787			D9S273		
Allele	PH2	Controls	Allele	PH2	Controls	Allele	PH2	Controls
4	8 (50%)	53 (29%)	3	2 (11%)	21 (12%)	4	1 (6%)	8 (5%)
5	8 (50%)	20 (11%)	4	1 (6%)	16 (9%)	5	1 (6%)	26 (15%)
Other	0 (0%)	113 (60%)	5	4 (22%)	40 (22%)	6	2 (11%)	38 (21%)
			6	3 (17%)	23 (13%)	7	2 (11%)	24 (14%)
			9	5 (28%)	38 (21%)	8	11 (6%)	62 (35%)
			10	3 (17%)	21 (12%)	9	1 (6%)	6 (3%)
			Other	0 (0%)	19 (11%)	Other	0 (0%)	14 (8%)
$P<0.0001$[a]			$P=0.8240$[a]			$P=0.3590$[a]		

[a] $P<0.05$ corresponds to a significant difference between patient and control allele frequencies

exon 6 alters an amino acid in the putative co-factor binding site.

Microsatellite linkage with the *GRHPR* gene in PH2 patients

Ten out of 11 patients studied by our group were homozygous for their mutation. This high proportion of homozygotes prompted us to assess the haplotype background of each patient in order to determine whether the patients were the offspring of related individuals.

The allelotype and haplotype data for the patients is summarized in Table 2. Data not shown in Table 2 include the heterozygosity of the control group: 86%, 76%, and 50% for markers D9S1874, D9S1787, and D9S273, respectively, compared with that for the patients of 0%, 36%, and 27%. The similar haplotypes (5,9,8; 5,5,8 and 5,10,8) generated for the most common mutation, 103delG (exon 2), suggest the presence of a common ancestral haplotype resulting from a founder effect (Table 2). This suggestion is supported by the finding that all patients share the same allelotype (5,5) at locus D9S1874. At D9S1787, the presence of three alleles co-segregating with the mutant allele represents a departure from the homozygous pattern at D9S1874. These data suggest that there has been at least one recombination event between D9S1874 and D9S1787 since formation of the founder allele.

The two pairs of North American siblings with the 103delG mutation studied in a previous report (Cramer et al. 1999; designated as CA2–5) had no personal knowledge of relatedness between their two families. The co-segregation of the mutated allele to the two haplotypes 5,10,8 and 5,5,8 supports the belief that these two families are not closely related. However, the finding that all patients with this mutation, except CA1, are homozygous for all markers, suggests they are all offspring of related individuals. Whether the parents are closely or distantly related to each other cannot be ascertained from these data. Because of heterozygosity at D9S1787 and D9S273, patient CA1 is most probably not the offspring of related individuals. Furthermore, the divergence of alleles for this patient (Table 2), including a further co-segregation of the mutant allele at D9S273, suggests that this patient's chromosomes have undergone more recombinations than that of the other patients.

Patient AA1, being homozygous for all markers, is also probably the offspring of related individuals, whereas patient InAs1, who is heterozygous for D9S1787 and D9S273, is most probably not.

The haplotypes constructed for the two patients (CIt1 and CIt2) with the identical mutation in exon 4 suggest that these two patients are not closely related (Table 2). However, the similarity of the chromosomes indicates the retention of a partial founder haplotype, with evidence of at least one recombination. The homozygosity of patient CIt1 suggests that he is the offspring of related individuals, whereas the heterozygosity of CIt2 at D9S1787 and D9S273 suggests otherwise for this patient. Likewise, CIt3 does not appear to be the product of related persons.

Allele frequencies are tabulated in Table 3. The frequency of patient alleles (the alleles of siblings were not counted twice), were compared with the frequency among the control group. Table 3 shows that the allele frequencies for D9S1874 differ significantly, whereas for D9S1787 and D9S273, there are no significant differences. These data indicate that D9S1874 is in linkage disequilibrium with the *GRHPR* gene, whereas D9S1787 and D9S273 are not.

Discussion

In this report, we have identified five novel mutations in the *GRHPR* gene in PH2 patients of African American, Caucasian Italian, and Indian Asian background. We have also detected the previously published exon 2 mutation in two more patients of North European (Caucasian American and German) descent. Ten out of the 11 patients studied by our group were homozygous for their given mutation. The spectrum of mutations identified included deletions, a nonsense transition, a missense transition, and missense transversions, resulting in either a truncated protein or a protein with a missense mutation or a splicing error. The missense mutations were found to result in a dysfunctional GRHPR protein in vitro. Therefore, it appears that all six mutations discovered thus far are disease-causing mutations. This result further confirms previous evidence (Cramer et al. 1999) indicating that mutations in the

GRHPR gene form the genetic basis of PH2. Furthermore, the heterozygosity of asymptomatic parents supports the original belief (Williams and Smith 1968) that PH2 is inherited in an autosomal recessive pattern.

The presence of disease-causing missense mutations is useful for studying the structure and function of the human GRHPR protein molecule. The G494A (exon 6) mutation alters an absolutely conserved amino acid that is involved in the putative co-factor binding site of the *H. methylovorum* enzyme (Fig. 1). The loss of protein function therefore confirms the importance of this amino acid in the human protein in vitro. The T965G (exon 9) mutation does not affect any of the putative co-factor or substrate binding sites (Fig. 1). However, the methionine is conserved in *H. methylovorum* and conservatively substituted with a valine and leucine in *Cucurbita maxima* and *Schizosaccharomyces pombe*, respectively (Cramer et al. 1999). Furthermore, recent completion of the *Drosophila melanogaster* euchromatic genomic sequence (Adams et al. 2000) has allowed us to identify the putative GRHPR homolog for this organism. Comparison with other GRHPR sequences has shown this methionine to be conserved in *D.melanogaster* (S. D. Cramer, unpublished). The substitution of an arginine, which represents a charge change, is therefore a significant alteration, and the loss of protein function confirms the importance of this residue. The mechanism by which protein function is lost can be speculated about on the basis of the crystallographic studies of the *H. methylovorum* enzyme. The position of this residue in the peripheral catalytic domain of the native monomer (Goldberg et al. 1994) suggests that it is not involved in the dimerization domain. Instead, the structural abnormality introduced by the arginine could destabilize the structure and trigger proteolytic degradation, interfere with the protective role of molecular chaperones (if required), and allow aggregation of the unfolded or partially folded protein (Hartl et al. 1994; Scriver and Waters 1999), or if the dimer is stable, it could simply interfere with the role of the catalytic subunit.

The functional importance of these two mutant residues demonstrates the potential contribution of missense mutations toward an understanding of the structure and function of the human GRHPR protein. This knowledge could aid the design of new therapeutic approaches to PH2.

The unusually high occurrence of homozygous mutations among the patients studied (10 out of 11) prompted us to investigate the possibility that the patients are the offspring of related individuals. Furthermore, haplotyping was applied to investigate whether common mutations present in unrelated individuals had arisen independently or were the result of a founder effect. Genotyping with microsatellite markers suggested that 7 out of 11 patients were the offspring of related individuals. The degree of relatedness could not be determined from this analysis; however, the parents of CA2–5 denied any knowledge of consanguineous marriages within their family pedigree. The remaining four patients did not appear to be the offspring of related parents. This, coupled with evidence that some patients of the same nationality with identical mutations are unrelated (exon 2 and exon 4 mutations), suggests that the disease alleles have undergone recombination and are not limited to a small inbred population. This supports observations that the PH2 patients reported in the literature are drawn from different populations. Therefore, this presence of disease alleles in "outbred" populations should encourage a more rigorous approach of the use of discriminatory diagnostic assays, which currently appear to be under-utilized (Petrarulo et al. 1998; Leumann and Hoppe 1999).

The data for the 103delG (exon 2) mutation suggest that historical recombinations around D9S1787 have disrupted a founder mutation that is most likely of North European descent. Furthermore, comparison of the German and American alleles at D9S1787 indicates that recombination has also occurred after segregation of the mutation into these two populations. This evidence of recombination suggests that the founder mutation is probably several generations old.

The homozygosity at D9S1874 for all the PH2 patients (Table 2), coupled with the significant differences in allele frequencies between patients and controls at this marker only (Table 3), implies linkage of the disease with D9S1874. Further evidence of linkage with this marker may prove useful for molecular diagnostics of PH2.

In conclusion, this report provides further evidence for the genetic basis of PH2. The identification of mutations in the North American and Italian populations could prove helpful in genetic counseling, molecular diagnostics, and the development of therapeutic approaches toward PH2. In addition, elucidation of the structural and functional characteristics of the human protein by use of these and as yet undiscovered mutations could potentially be of use in the development of therapeutic approaches. Furthermore, the presence of disease alleles in "outbred" populations, coupled with previous evidence of under-diagnosis, should prompt a more diligent application of discriminatory diagnostic assays.

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