Mutations in the protoporphyrinogen oxidase gene in patients with variegate porphyria

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Variegate porphyria (VP) is an acute hepatic porphyria with autosomal dominant inheritance due to a partial deficiency of protoporphyrinogen oxidase (PPOX) activity. The molecular defect responsible for VP was investigated by sequencing PPOX gene coding sequence from four patients in three unrelated VP families of French Caucasian origin. In a first patient, a point insertion of a G at position 1022 of the cDNA, produced a frameshift resulting in a premature stop codon. In three other patients from two unrelated families we found a missense point mutation leading to glycine to arginine substitution (G232R) in exon 7. This Gly232 appears to be a strictly conserved residue through evolution. In one VP family, we observed the cosegregation of the G232R missense mutation and the deficient PPOX activity. The mutations reported here are the first to be described in patients with VP and support the conclusion that PPOX gene defects are disease causing mutations in human variegate porphyria.

INTRODUCTION

Variegate porphyria (VP) is an autosomal dominant disease with incomplete penetrance due to a partial deficiency of protoporphyrinogen IX oxidase (PPOX, EC 1.3.3.4). PPOX is a mitochondrial enzyme (1) that catalyses the oxidation of protoporphyrinogen to protoporphyrin IX, the penultimate step in the haem biosynthetic pathway. Clinical features of VP resemble those found in other acute hepatic porphyrias, acute intermittent porphyria (AIP) and hereditary coproporphyria (HC) (2). Biochemically, increased excretion of porphyrin precursors, δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) in urine and of protoporphyrin and, to a lesser extent, coproporphyrin in faeces is observed (2). In agreement with the dominant inheritance in VP, PPOX activity is decreased to 50% of normal in all tissues tested from VP patients as well as from asymptomatic carriers of the gene defect (3–5).

Recently, human cDNA encoding PPOX has been sequenced (6) and the human PPOX gene has been cloned, assigned by FISH to chromosome 1 and its exon/intron organization was characterized (7). More recently, VP gene was mapped by linkage analysis to the same position on chromosome 1 (8) in contrast to a previous report on chromosome 14 (9). In this study, abnormalities in PPOX gene from VP patients have been defined.

RESULTS

Determination of the mutations

Two overlapping PCR amplified fragments of expected size, that covered all PPOX cDNA coding sequence, were obtained from normal controls and from the four VP patients cDNAs. Direct sequencing of PCR products from patient P1 revealed a heterozygous insertion of a G after nucleotide 1022 (1022insG; Fig. 1). The insertion is responsible for a frameshift and produces a TGA stop codon 31 codons downstream of the inserted nucleotide. In patient P2 and in the two related patients P3 and P4, direct cDNA sequencing revealed a G to C transversion in the heterozygous form at nucleotide 971 (G971C; Fig. 1). The base change results in a glycine to arginine substitution at position 232 (G232R) in the deduced amino acid sequence. This glycine residue appears to be strictly conserved through evolution (Fig. 2). The mutation creates a new Acyl restriction site, which was used to analyse cDNAs from 48 unrelated normal subjects; all were found to be negative for the presence of the restriction site. No other abnormality was found in the PPOX coding sequence from the four VP patients.

Both mutations were located in a cDNA region that corresponds to exon 7 of the PPOX gene. They were further confirmed at the genomic level by direct sequencing of a PCR amplified fragment from genomic DNA that included exon 7. The G232R mutation could also be detected on the same DNA fragment using Acyl restriction analysis. Accordingly, both VP patients P2 and P3 were found to be positive for the presence of the restriction site, i.e. the presence of the mutation in the heterozygous state (not shown).

VP family study

Acyl restriction analysis performed on cDNA permitted the analysis of the segregation of the G232R mutated allele through three generations in the family of patients P3 and P4. Data shown
Figure 1. Partial sequence analysis of PCR-amplified PPOX cDNA. P1: Insertion of a G, in the heterozygous state, between positions 1022–1023 of the cDNA from VP patient P1. P2: G to C transversion at nucleotide 971. The mutation was found in the heterozygous form in VP patient P2 and in the two related patients P3 and P4. N: Normal sequence.

Figure 2. Comparison of nucleotide deduced amino acid sequences from codon 217 to 246 of human PPOX (HS) with that of mouse (MM), Hem14 gene product from Saccharomyces cerevisiae (SC-HEM14) and HemY gene product from Bacillus subtilis (BS-HEMY). Identical residues are indicated by asterisks and quasi-identity, including conservative substitutions, is indicated by dots. Dashes indicate spaces introduced to allow maximum alignment of identical residues (Clustal W1.5, multiple sequence alignment program).

in Figure 3 illustrate the cosegregation of the mutation and lymphocyte PPOX activity in a pedigree of 14 members. The presence of a digested fragment was always correlated with a deficiency in PPOX activity.

Intragenic dimorphism
While searching for mutations in PPOX cDNA, we identified and characterized as a biallelic polymorphism, a G to A transition at nucleotide position 1188 (1188G/A). This transition occurred in exon 9 of the PPOX gene, leading to arginine to histidine substitution at codon 304 (R304H) and creating a BssSI restriction site. Allele frequencies were calculated from the genotypes obtained by BssSI restriction analysis of 54 normal unrelated French Caucasians: A1(G), 0.56; A2(A), 0.44. In the VP family studied above (Fig. 3), the 1188G/A dimorphism, detected by BssSI, was not associated with the mutated allele (not shown).

DISCUSSION
The human PPOX cDNA was cloned by in vivo complementation using a hemG mutant of Escherichia coli (6), on the basis that the protein product of hemG gene has PPOX activity, although it may be structurally different from mammalian PPOX (6,10,11). Subsequently, the VP gene was mapped to the same position on chromosome 1 as PPOX gene (8,9). Data in the present report give strong evidence that two different mutations found in the PPOX gene coding sequence from four patients in three different families with VP, are responsible for the decrease in PPOX activity and the disease.

In VP patient P1, an insertion of a G was found in the cDNA sequence (1022insG) at the heterozygous state. No other abnormality was found in the coding sequence and the insertion mutation was confirmed in exon 7 of the PPOX gene. The G insertion occurred after a stretch of six G repeats and therefore could be the result of strand slippage during DNA replication (12). The resulting frameshift and premature stop codon may be responsible for the synthesis of a truncated protein with no catalytic activity and shortened half-life.

In VP patient P2 and in the two related patients P3 and P4, a 971G to C transversion was identified in the cDNA sequence, which results in amino acid substitution G232R. The base change is thought to be the mutation responsible for VP in these cases for the following reasons: there was no other abnormality found in the coding sequence of PPOX gene from the three patients and the base change was never found in 48 unrelated normal individuals that were subjected to Acyl restriction analysis. The G insertion occurred after a stretch of six G repeats and therefore could be the result of strand slippage during DNA replication (12). The resulting frameshift and premature stop codon may be responsible for the synthesis of a truncated protein with no catalytic activity and shortened half-life.

A common intragenic dimorphism (R304H) is also reported, which may be useful for linkage analysis in VP families. It is noteworthy that the G232R mutation occurred in two unrelated VP families of French Caucasian origin and that both mutations...
reported here were located in exon 7 of the PPOX gene. This could be in contrast with the wide molecular heterogeneity of the mutations that was shown in other forms of acute porphyrias (AIP, HC) (15,16). However in VP, as the genomic PPOX structure is only recently available, the search for mutations was restricted to the PPOX cDNA sequence. Moreover, in the studied VP family, the R304H dimorphism did not segregate with the status of the pedigree members, ruling out a possible functional effect in the VP phenotype outcome.

MATERIALS AND METHODS

Patients and controls

Four patients (P1–P4) from three unrelated French Caucasian families with VP were studied. Diagnosis of VP was made based on a clinical history of at least one acute attack and/or typical cutaneous lesions associated with increased excretion of ALA and PBG in urine and of protoporphyrin IX in faeces. A 50% decreased PPOX activity in lymphocytes was documented for all four patients (P1, 2.0; P2, 3.0; P3, 2.1; P4, 2.8; mean normal controls value ± SD: 4.8 ± 0.6 nmol protoporphyrin/h per mg protein).

In one VP family with two patients (P3 and P4), asymptomatic carriers could be identified through three generations by decreased lymphocytes PPOX activity (5). From this VP family, a large pedigree of 14 relatives, including patients, carriers and normal subjects, was available for molecular investigation (Fig. 3). Peripheral blood was collected from VP patients, members of the VP family and from 54 unrelated normal controls of Caucasian origin.

Cell culture

Epstein–Barr virus transformed lymphoblastoid cells line from VP patients and normal controls were established as described previously (17).

RNA extraction and in vitro amplification of cDNA by polymerase chain reaction (PCR)

Total cellular RNA was extracted with TRIzol™ (Gibco-BRL, Life Technologies Inc., Gaithersburg, MD, USA) according to the manufacturer’s recommendations. cDNA was obtained by reverse transcription of total RNA using oligo(dT) as a primer.

PPOX cDNA was amplified as two overlapping fragments using two sets of specific primers, respectively, POX1-S and POX3-AS (793 bp fragment) and POX6-S and POX5-AS (896 bp fragment). Five µl of the cDNA preparation was added to 45 µl of PCR solution containing 1 U of Taq polymerase (Beckman Inc., Fullerton, CA, USA), 50 mmol/l KCl, 10 mmol/l Tris–HCl pH 8.5, 1.5 mmol/l MgCl₂, 200 mmol/l of each dNTP, 20 pmol of each primer. A hot start PCR was performed with an initial denaturation step at 94°C for 1 min. The reactions were carried out in a DNA thermocycler (Hybaid, Teddington, UK) as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and elongation at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

cDNA sequencing

The PCR products were purified using the Wizard PCR purification system (Promega-Biotech, Madison, WI, USA) and direct PCR sequencing was performed in both orientations using a fmol DNA sequencing kit (Promega-Biotech) with PPOX specific oligonucleotides as sequencing primers.

Confirmation of the mutations at the genomic level

Genomic DNA was extracted from peripheral blood as previously described (18). A PPOX DNA sequence comprising exon 7 was amplified with a set of specific primers, POX6-S, located in exon 6 and gPOX7-AS, located at the exon 7/intron 7 junction. Amplification was performed under the same hot start PCR conditions as described above for cDNA and a fragment of approximately 640 bp length was obtained. The PCR product was purified and sequenced as described above and using the sequencing primer gPOX7SEQ-AS and an annealing temperature set of 62°C.

DNA restriction analysis

AcyI restriction analysis was performed as follows: 10 µl of amplified product were incubated with 1 U of AcyI (Boehringer Mannheim, GmbH, Biochemica, Mannheim, Germany) in the appropriate buffer at 50°C during 5 h.

The restriction studies using BssSI were performed on a cDNA fragment of 375 bp length obtained after amplification using POX6-S and POX4-AS as specific primers. Ten µl of amplified cDNA were incubated with 1 U of BssSI (New England Biolabs Inc., Beverly, MA, USA) in the appropriate buffer at 37°C for 3 h. Digestion produces two fragments of 334 and 41 bp in length, respectively.

Oligonucleotides used

Oligonucleotides were obtained from Genset (Paris, France) with the following sequences:

POX1NC-S: 5′-CAAGCAGAGCACCAGAGACTCA
POX1-S: 5′-CTCCAGCTCTCCTCTCCCTCA
POX2-AS: 5′-CCTCAGCCGCAAGCACCAGAACA
POX3-AS: 5′-TACTAGTCAGGGTGGTTC
POX4-AS: 5′-TCACACAGCTACAGACACCT
POX4SEQ-AS: 5′-TAGACCCAGCACCTCCAGCA
POX5-AS: 5′-GCTCCAGCAATTTTTAATTT
POX5SEQ-AS: 5′-TCCAGCAATTTTTATTTT
POX6-AS: 5′-CCCCATGTCCATATTTCTA
POX6SEQ-AS: 5′-GGTGGCAGTGTTCTTTCCTCA
gPOX7SEQ-AS: 5′-TGGAGGCTGAGCCCACAGACT
gPOX7AS-7: 5′-tacctccaggccgccccttct
POX7SEQ-AS: 5′-TACACGCAACTCCCTCCTAG
POX8SEQ-AS: 5′-GAACTGGAATGCCACTAA

The exon sequence is shown in upper case letters and the intron sequence is shown in lower case letters.
ABBREVIATIONS

AIP: acute intermittent porphyria; HC: hereditary coproporphyria; VP: variegate porphyria; FISH: fluorescent in situ hybridization; PPOX: protoporphyrinogen oxidase.

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