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High Frequency of Mutations in Exon 10 of the Porphobilinogen Deaminase Gene in Patients with a CRIM-positive Subtype of Acute Intermittent Porphyria

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Summary

Acute intermittent porphyria (AIP) is an autosomal dominant disease characterized by a partial deficiency of porphobilinogen (PBG) deaminase. Different subtypes of the disease have been defined, and more than 10 different mutations have been described. We focused our study on exon 10, since we previously found that three different mutations were located in this exon and that two of them seemed to be relatively common. We used denaturing gradient gel electrophoresis (DGGE) after in vitro amplification to detect all possible mutations in exon 10 in 41 unrelated AIP patients. In about one-fourth of these patients we could distinguish three abnormal migration patterns, indicating the presence of various mutations. Additional sequencing demonstrated the presence of three different single-base substitutions. Two of these mutations had already been described. A third one consisted of a C-to-T transition located at position 499 of the PBG deaminase mRNA and resulted in an Arg-to-Trp substitution. All three mutations were found in patients with cross-reacting immunological material (CRIM)–positive forms of AIP. The high frequency of these mutations make DGGE analysis of exon 10 a useful approach allowing the direct detection of the DNA abnormality in most of the families with the CRIM-positive subtype of AIP.

Introduction

Acute intermittent porphyria (AIP) is an autosomal dominant disease which is defined by a partial deficiency of porphobilinogen (PBG) deaminase (hydroxymethylbilane synthase; E.C.4.3.1.8), the third enzyme of the heme biosynthetic pathway. The clinical manifestations are characterized by acute attacks of neurological dysfunctions, with abdominal pains, hypertension, tachycardia, and peripheral neuropathy (Kappas et al. 1989).

The molecular heterogeneity of the mutations producing AIP was suggested by DNA studies at the PBG deaminase locus, which demonstrated that AIP mutations were associated with different restriction haplotypes (Llewellyn et al. 1987; Lee et al. 1988; Scobie et al. 1990). We have previously identified two different mutations in exon 1 of the PBG deaminase gene, accounting for a subgroup of AIP families in which the expression of the enzymatic defect is restricted to non-erythropoietic cells (Grandchamp et al. 1989*b*, 1989*c*). Moreover, several phenotypic subtypes at the protein level have been described. In the more common forms of AIP, in which the PBG deaminase deficiency is present in all cell types, different investigators have documented the existence of two subgroups of patients on the basis of measurement of cross-reacting immunological material (CRIM) in erythrocytes, groups referred to as "CRIM-negative" and "CRIM-positive" mutations (Desnick et al. 1985; De Rooij et al. 1987).

Recent studies in our laboratory identified two mutations responsible for CRIM-positive forms of the

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disease (Delfau et al. 1990). These single-base substitutions, both located in exon 10 of the PBG deaminase gene, accounted for the enzymatic abnormality in six of eight patients with this subtype of AIP. In the CRIM-negative group, five different mutations were described, and each of them was detected in only one family (Scobie et al. 1990; Delfau et al. 1991). One of these mutations is also located in exon 10. The high frequency of mutations in exon 10 of the PBG deaminase gene prompted us to establish the overall frequency of the mutations located in this exon in AIP patients. For this purpose, we used denaturing gradient gel electrophoresis (DGGE) as a mean of detecting all possible mutations in this exon after in vitro DNA amplification (Sheffield et al. 1989).

Patients and Methods

Patients

A total of 41 unrelated patients from The Netherlands were studied. All of them were AIP patients with unknown mutations, 10 patients with CRIM-positive and 31 with CRIM-negative forms of AIP. The diagnosis was established on the basis of clinical symptoms of the disease accompanied by increased urinary excretion of PBG and 5-aminolevulinic acid and by normal fecal porphyrin excretion. Diminished activity of PBG deaminase was also observed in erythrocytes of these patients. CRIM determination was performed according to a method described elsewhere (De Rooij et al. 1987; Grandchamp et al. 1989*a*).

DNA Amplification

DNA was prepared from peripheral blood leukocyte samples according to the method of Higuchi (1989). DNA amplifications were carried out using the PCR according to the method of Saiki et al. (1988). DNA samples were amplified in 50-μl reaction mixtures containing 200 ng of genomic DNA, 50 mmol KCl/liter, 10 mmol Tris pH 8.3/liter, 1.5 mmol $MgCl_2$/liter, 0.2 mmol dATP/liter, 0.2 mmol dCTP/liter, 0.2 mmol dGTP/liter, 0.2 mmol dTTP/liter, 25 pmol of each primer, and 1 unit of *Taq* polymerase (Stratagene). The cycling reaction was performed in a programmable heating block (DNA thermal reactor; Hybaid). The DNA samples were initially denatured for 3 min at 95°C, and then, with each cycle consisting of 15 s at 95°C, 30 s at 56°C, and 40 s at 72°C, the PCR was carried out for 30 cycles. To allow the formation of heteroduplexes, the incubation mixture was heated at 95°C for 5 min and was allowed to reanneal for 5 min at 72°C after completion of the last cycle. Two primers were designed to match a genomic sequence in intron 9 for the sense primer (Phi9, 5′-CCGACACTGTGGTCCTTAGCAA-3′) and in intron 10 for the antisense primer with the addition of a 30-base clamp (Phi10 clamp, 5′-CGCCCGGCGGC-CCTCCCGCGGGCGGCGGGCTGGGGATGACT-GTAAGGCAGAA-3′), allowing the amplification of a 240-bp segment containing exon 10 of the PBG deaminase gene.

DGGE

DGGE was performed according to the method of Myers et al. (1987). Computer analyses were carried out using Melt 87 meltmap programs according to the method of Lerman and Silverstein (1987). Each amplified sample (15 μl) was mixed with loading buffer and electrophoresed in an 8% polyacrylamide gel with a linear gradient of 50%–80% denaturant (100% denaturant = 7 mol/liter urea/40% formamide) at 150 V for 3 h. The gel was stained in ethidium bromide and was photographed under UV light with Polaroid film type 667.

DNA Sequencing

Direct DNA sequencing was carried out after PCR amplification, according to a method described by Hutman et al. (1989). Primers used for amplification were Phi9 and Phi10 (without clamp), the former primer being biotinylated. After amplification, single-stranded DNA was obtained by mixing 25 μl of PCR products with 30 μl of Dynabeads M-280 streptavidin (Dynal, Norway), denaturing the DNA with 0.15 mol NaOH/liter, and washing twice with water. Sequencing was performed using a sequenase kit (U.S. Biochemical). The sequencing primer was the same as the amplification primer (Phi10), and internal labeling was obtained with α^{-35} S-dATP (Amersham).

Site-directed Mutagenesis

A normal human cDNA sequence cloned in the plasmid vector PGEM3 (Promega Biotech) was modified to introduce the C-to-T mutation at position 499 (see Results) by using site-directed mutagenesis. The PCR was performed according to a method described by Higuchi (1990), except that the thermostable polymerase pfu (Stratagene) was used instead of *Taq* polymerase. In brief, two partially overlapping fragments were synthesized using two sets of primers, one primer from each pair targeting the desired sequence to be

changed. The following sets of primers were used: PheNco, 5′-ATGTCTGGTAACGGCAATGCGG-3′ and Phe499AS, 5′-GGTTTCCCCGAATACTCCT-3′; and Phe499, 5′-AGGAGTATTCGGGGAAACCT-CA-3′ and Phe15Hind, 5′-CATCTGTGCCCCACA-AACCA-3′. The reaction mixtures contained 1 ng of plasmid, 25 pmol of each primer, 0.2 mmol each dNTP/liter, and 1 unit of pfu polymerase (Stratagene) in 50 µl of 1 × buffer 1 from the manufacturer.

After an initial denaturation for 3 min at 94°C, 30 cycles of PCR were performed, each cycle consisting of 15 s at 95°C, 1 min 30 s at 44°C, and 1 min 10 s at 72°C. The resulting PCR products were gel purified on a 3% Nusieve gel and were mixed in a 1:1 molar ratio, and about 5 ng of each fragment was added to a reaction mixture of 50 µl containing containing 1 × buffer 1 (Statagene), 0.2 mmol each dNTP/liter, and 1 unit of pfu DNA polymerase. After five cycles, each consisting of 15 s at 95°C, 1 min at 48°C, and 1 min 30 s at 72°C, primers PheNco and Phe15Hind were added (25 pmol each), and the PCR was allowed to process for 25 additional cycles, each consisting of 15 s at 95°C, 40 s at 55°C, and 1 min 40 s at 72°C.

Bacterial Expression of PBG Deaminase

cDNA fragments were phenol extracted and ethanol precipitated. The purified fragments were digested with *Nco*I and *Hin*dIII, were purified through spun-down chromatography using Sephacryl S200, and were cloned into the bacterial expression vector PKK 233-2. The sequence of cloned cDNA was verified, and PBG deaminase activity was determined from bacterial cultures according to a method described elsewhere (Delfau et al. 1990). Both separation of normal and mutated human PBG deaminase from the enzyme from *Escherichia coli* and western blot analysis were performed according to methods described elsewhere (Delfau et al. 1990).

Results

A rapid screening for the presence of mutations in exon 10 of the PBG deaminase gene was performed by DNA analysis of 41 unrelated AIP patients from The Netherlands by using PCR amplification and subsequent DGGE analysis of the PCR products. The melting map of this fragment is represented on figure 1. Of the 41 patients that we studied, 8 gave rise to amplified products with an abnormal melting pattern (fig. 2 and data not shown). In each case, in addition to a band corresponding to the normal fragment, three additional bands were observed, corresponding to a fragment with a mutated sequence and to two heteroduplexes. Three different patterns of migration were observed, suggesting that at least three different muta-

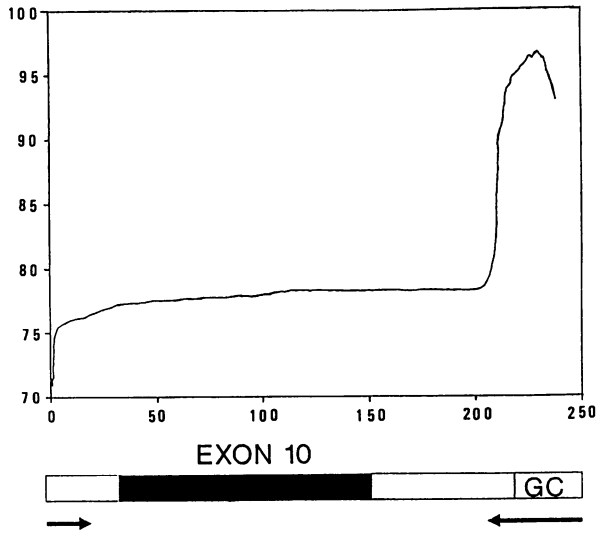

Figure 1 Melting map of the amplified fragment containing exon 10 of the PBG deaminase gene with a GC-clamp at its 3′ end. The T_m is plotted as a function of the nucleotide sequence position of the DNA fragment. The line shows the temperatures at which each base pair is in 50:50 equilibrium between the helical and melted configuration.

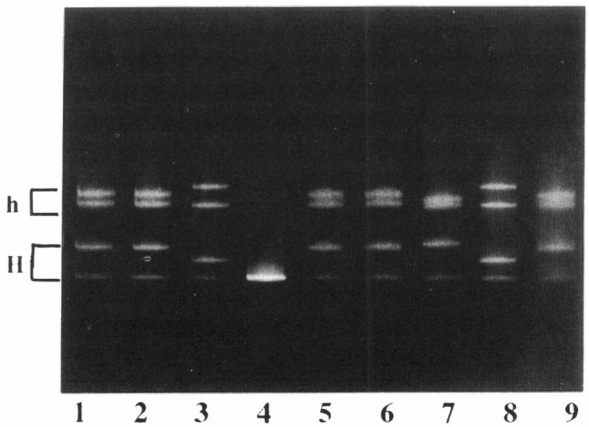

Figure 2 Analysis of exon 10 of the PBG deaminase gene by DGGE. Ethidium bromide–stained gels after DGGE of amplified fragments from AIP patients (lanes 1–3 and 5–9) heterozygous for three different mutations in exon 10 and from a normal control (lane 4) show mutations 500 (lanes 1, 2, 5, 6, and 9), 518 (lanes 3 and 8), and 499 (lane 7). The location of the mutations is numbered from the initiating codon in the PBG deaminase mRNA. h = Heteroduplexes; and H = homoduplexes.

tions could be detected. The nature of these mutations was determined by direct sequencing of amplified products. Two mutations consisted of G-to-A transitions, one at position 500 and one at position 518 of the coding sequence of the PBG deaminase mRNA. Such mutations had previously been found in other patients in our laboratory (Delfau et al. 1990). In the present study, six patients had the mutation at position 500, and two had the mutation at position 518. A previously undescribed type of mutation was found in another patient and consisted of a C-to-T transition at position 499 of the open reading frame, resulting in an arginine-to-tryptophan amino acid substitution in the abnormal protein. The same mutation was also detected in two relatives of the patient. In order to evaluate the functional consequences of this mutation, the base change was introduced into a normal cDNA sequence by site-directed mutagenesis, and the mutated cDNA was cloned in the expression plasmid PKK 233-2. Of 10 clones that we tested, all of them failed to express the human enzyme activity, although clones containing the normal cDNA overexpressed PBG deaminase activity. After verification of its nucleotide sequence, one mutated cDNA clone was further studied. The specific activity of the abnormal enzyme was 4% of the normal human PBG deaminase activity (mean of four independent determinations) when measured at the optimal pH of the normal enzyme (pH 8). A western blot analysis of the bacterial proteins by using a specific antiserum against PBG deaminase revealed the presence of a stable immunoreactive enzyme (fig. 3). All the patients whom we found with a mutation in exon 10 during the present study presented CRIM-positive forms of AIP (table 1), and, conversely, only 2 of 10 patients with the same subtype of the disease had no abnormality in this exon; in these 2 patients, the absence of a base change in exon 10 was confirmed by direct sequencing of PCR products. In the CRIM-negative group, none of the patients presented any detectable abnormality in exon 10.

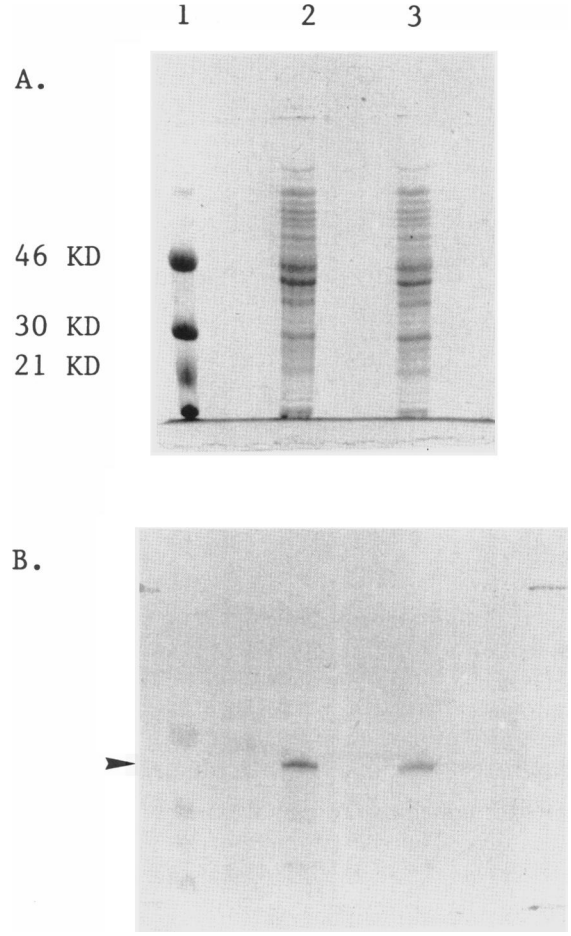

Figure 3 Electrophoretic analysis of samples containing human PBG deaminse. *A*, Samples submitted to SDS gel electrophoresis using homogeneous Phastgels 12.5% (Pharmacia, Sweden). The gel was silver stained. Lane 1, Molecular-weight markers. Lane 2, lysate from *Escherichia coli* cells expressing a normal human nonerythroid form of PBG deaminase. Lane 3, Lysate from *E. coli* expressing mutant M499 of the nonerythroid form of PBG deaminase. *B*, Western blot analysis of PBG deaminase. The samples are identical to those in panel *A*. After migration, the proteins were electrophoretically transferred to a nitrocellulose membrane, and human PBG deaminase was detected by using specific antibodies. The arrowhead indicates the position of PBG deaminase.

Discussion

Previous work has indicated that AIP is a heterogeneous disease at the molecular level and that point mutations, rather than large deletions, are likely to account for most of the cases (Llewellyn et al. 1987). Eleven different mutations have already been identified (Grandchamp et al. 1989*a*, 1989*b*, 1989*c*; Delfau et al. 1990, 1991; Scobie et al. 1990). All known mutations are readily detectable by hybridization of PCR-amplified genomic sequences by allele-specific oligonucleotides. However, the efficiency of this approach is limited by the extent of the heterogeneity of the mutations. For this reason, we used DGGE as an alternative approach to detect all possible mutations in a defined DNA fragment after PCR amplification (Myers et al. 1987; Sheffield et al. 1989). Elsewhere we have used this method to detect mutations in exon

Table 1

CRIM Ratios in 10 CRIM-positive AIP Patients with Different Mutations in Exon 10 of PBG Deaminase

	No. of Patients	CRIM Ratio
Mutation 499	3	2.07 ± 0.15
Mutation 500	6	2.89 ± 0.90
Mutation 518	2	1.84 and 1.75
Not detected	2	1.67 and 2
Control	10	1 ± 0.15

1 of the PBG deaminase gene (Bourgeois et al. 1992). The major advantages of the DGGE approach are its simplicity, since neither hybridization nor enzymatic digestion is required, and, most important, the possible ability to detect mutations in the domain under investigation, mutations which may be different from already known mutations. About one-fourth of the unrelated patients whom we studied by DGGE yielded an abnormal pattern of migration and were shown to carry single-base substitutions in exon 10. Two of these mutations have already been described (Delfau et al. 1990). A third one consisted of a C-to-T transition located at position 499 of the PBG deaminase mRNA and changed the same codon as did the previously described G-to-A transition at position 500. The functional consequences of this base change were further investigated by introducing the 499 mutation into a normal PBG deaminase cDNA by site-directed mutagenesis and then cloning the mutated cDNA into a bacterial expression vector. As previously reported for other CRIM-positive mutations (Delfau et al. 1990), the abnormal cDNA was able to direct the synthesis of an immunodetectable but catalytically inactive protein in the bacterial system. All three mutations reported here were found in patients with CRIM-positive forms of AIP. Only two other patients with the same phenotypic subtype of the disease had no abnormality of exon 10. CRIM-positive mutations have been found in 15%–25% of AIP families (Desnick et al. 1985; De Rooij et al. 1987; present study). It is noteworthy that the newly found mutation leads to an arginine-to-tryptophan amino acid change in the mutated protein, involving the same arginine as does the G-to-A transition located at position 500. This confirms the importance of this amino acid residue for the catalytic activity of the enzyme. This role may involve the binding of either PBG or one of the polypyrrole intermediates during the stepwise polymerization of four PBG molecules. It is of interest that this arginine is highly conserved between species for which sequence data are available, including rat, mouse, *Escherichia coli,* and *Euglena gracilis* (Thomas and Jordan 1986; Stubnicer et al. 1988; Beaumont et al. 1989; Sharif et al. 1989). Other lines of evidence suggest that arginine residues may be involved in substrate binding in other enzymes of the heme biosynthetic pathway (Dailey et al. 1986). Taken together with previous results from our laboratory (Delfau et al. 1990), our data suggest that a large majority of CRIM-positive patients (12 of 16 patients) have single-base substitution in exon 10 of the PBG deaminase gene, with a higher frequency of the G-to-A transition at position 500 (10 patients). However, since the majority of the patients in our studies originated from The Netherlands and from France, the frequency of these mutations must be evaluated in CRIM-positive patients from different origins. In the CRIM-negative forms of AIP, most of the mutations reported so far consist of point mutations resulting in single amino acid changes (Delfau et al. 1990, 1991), premature stop codons (Scobie et al. 1990), or splicing defects (Grandchamp et al. 1989*a*, 1989*b*, and 1989*c*). Such mutations could potentially be detected by using DGGE analysis of PCR products containing different exons and exon-intron junctions of the gene. This will allow evaluation of the extent of the diversity of the mutations responsible for AIP.

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