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Four Novel PEPD Alleles Causing Prolidase Deficiency

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Summary

Mutations at the PEPD locus cause prolidase deficiency (McKusick 170100), a rare autosomal recessive disorder characterized by iminodipeptiduria, skin ulcers, mental retardation, and recurrent infections. Four PEPD mutations from five severely affected individuals were characterized by analysis of reverse-transcribed, PCR-amplified (RT-PCR) cDNA. We used SSCP analysis on four overlapping cDNA fragments covering the entire coding region of the PEPD gene and detected abnormal SSCP bands for the fragment spanning all or part of exons 13–15 in three of the probands. Direct sequencing of the mutant cDNAs showed a G→A, 1342 substitution (G448R) in two patients and a 3-bp deletion (ΔE452 or ΔE453) in another. In the other two probands the amplified products were of reduced size. Direct sequencing of these mutant cDNAs revealed a deletion of exon 5 in one patient and of exon 7 in the other. Intronic sequences flanking exons 5 and 7 were identified using inverse PCR followed by direct sequencing. Conventional PCR and direct sequencing then established the intron-exon borders of the mutant genomic DNA revealing two splice acceptor mutations: a G→C substitution at position −1 of intron 4 and an A→G substitution at position −2 of intron 6. Our results indicate that the severe form of prolidase deficiency is caused by multiple PEPD alleles. In this report we attempt to begin the process of describing these alleles and cataloging their phenotypic expression.

Introduction

Prolidase (E.C.3.4.13.9) is a dipeptidase with absolute specificity for substrates containing proline or hydroxyproline at the carboxy-terminal. This ubiquitous cytosolic enzyme is a homodimer of 56-kD subunits and requires manganese for catalytic activity. Prolidase deficiency (PD) (McKusick 170100), an autosomal recessive disorder, is associated with iminodipeptiduria. The clinical phenotype is pleiotropic and includes skin ulcers, mental retardation, recurrent infections, and a characteristic facies. These features, however, are incompletely penetrant and highly variable in both age at onset and severity (Phang and Scriver 1989). The pathogenesis and cause of the phenotypic heterogeneity are poorly understood.

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The gene (PEPD) maps to chromosome 19p13.2 (Endo et al. 1989) and has 15 exons spanning >130 kb of genomic DNA (Tanoue et al. 1990*b*). The 2.3-kb prolidase mRNA encodes a polypeptide of 493 amino acids (Endo et al. 1989). Two mutations at the PEPD locus that cause PD have been identified elsewhere (Tanoue et al. 1990*a*, 1990*b*). Here we describe four novel mutations associated with PD.

Patients and Methods

Cell Strains and Growth Conditions

Fibroblast cultures were established from skin biopsies performed on five PD patients. Control cell lines were from foreskin biopsies obtained at infancy from the Montreal Children's Hospital. Biochemical data have been reported on patients and cell lines as follows: patient 1 (strain WG1082 of the Repository of Mutant Human Cell Strains, Montreal Children's Hospital) and patient 4 (WG1298; Boright et al. 1989); patient 2 (WG1194; Gray et al. 1983; Boright et al. 1989); patient 3 (WG1343; Jackson et al. 1975; Boright et al. 1989); and patient 5 (WG 1625; Bissonette et al. 1993). Cell lines WG1530 and WG1531 are from the parents of

Table 1

Oligonucleotide Primers and Probes

Primer Orientation	Primer Sequence	Primer Location[a]
Primer pairs for RT-PCR amplification:		
Sense	ATGGCGGCGGCCACCGGACC	Exon 1 (1–20)
Antisense	GGCAATCTCATCTACGTACT	Exon 4 (399–380)
Sense	AAGATCCATTCCAAGGAGCA	Exon 4 (328–347)
Antisense	GTCGAACAGGCACATATCCC	Exon 11 (828–809)
Sense	AGTGGTGAGAACTCAGCCGT	Exon 11 (739–758)
Antisense	CACGTCAATGCCCAGGAAGT	Exon 13 (1128–1109)
Sense	TGTTTATGCCTCACGGGCTT	Exon 13 (1085–1104)
Antisense	CCCGGGAAACAGCACTGTTT	Exon 15 (1639–1620)
Primer pairs for PCR amplification of genomic DNA:		
Sense	acgtcgcctgttctaatgat	IVS 4
Antisense	ctccccagagccaagcact	IVS 5
Sense	ttaaagtgtgggaccctctt	IVS 6
Antisense	tgggaatctgctttctgagg	IVS 7
Sense	TTCCTTAACCGCGAGGTCCT	Exon 14 (1300–1319)
Antisense	ACGCAGGTCAGCAGCTCTAT	Exon 15 (1403–1384)
Primer pairs for inverse PCR amplification:		
Sense	CCTCTGTCCTCCTCACTTTG	Exon 5 (422–441)
Antisense	GGACGCTGGCAATctagaag	Exon 5 (406)–IVS 4 (−7)
Sense	CACCCAGAGATCGTTGAGTG	Exon 7 (529–548)
Antisense	TGGTATTGTTGACTTCGAAC	Exon 7 (523–504)
Probes for allele-specific oligonucleotide hybridization:		
Normal	GCATCGAGGAGGACGTCG	3-bp deletion
Mutant	CGCATCGAGGACGTCGTG	3-bp deletion
Normal	GTTTTGGCGGGGTGCAGT	G→A, 1342
Mutant	GTTTTGGCAGGGTGCAGT	G→A, 1342

[a] Nucleotide positions in parentheses refer to cDNA positions (Endo et al. 1989).

patient 1. Fibroblasts were grown in T175 culture flasks and were fed Eagle's minimal essential medium (Eagle's MEM) containing 10% FCS, 1 mM pyruvate, and 20 mM sodium carbonate at 37°C in 5% CO_2 atmosphere.

Reverse Transcription-PCR Amplification (RT-PCR)

Total cellular RNA was isolated from fibroblasts (Chirgwin et al. 1979). Synthesis of cDNA was carried out using 5 μg of total cellular RNA in a final volume of 20 μl, containing 20 pmol of prolidase mRNA–specific downstream antisense oligonucleotide primers (table 1), 10 units of human placental ribonuclease inhibitor (BRL), 200 units of M-MLV reverse-transcriptase (BRL), and 1 mM dNTPs (Pharmacia) in reaction buffer (50 mM Tris-HCL pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT). One-tenth of the total cDNA product was subjected to PCR amplification in 100 μl of reaction mixture (25 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, and 200 μM dNTPs) containing 100 pmol of each primer and 2.5 units of *Taq* DNA polymerase. The PCR conditions were as follows: an initial 7-min incubation at 95°C, followed by 35 PCR amplification cycles (1 min at 95°C, 1 min at 55°C–59°C, and 1–2 min at 72°C) and a final 7-min incubation at 72°C. The RT-PCR products were isolated by agarose gel electrophoresis and were phenol extracted and directly sequenced.

SSCP Analysis

Four overlapping amplified cDNA fragments covering the entire coding region of PEPD were prepared from control skin fibroblast cells and three PD skin fibroblast cell lines. SSCP analysis (Orita et al. 1989) was performed on restriction fragments (<300 bp) of these RT-PCR products. Five microcuries of α-^{32}P-dCTP (NEN) were added to PCR reaction mixtures in a total volume of 25 μl. Electrophoresis was performed on a 6% polyacrylamide gel containing 10% glycerol and 1 × TBE (100 mM Tris, 90 mM boric acid, and 1 mM EDTA) at room temperature at 4 W for 16–18 h. The gel was then dried for autoradiography.

Genomic DNA Amplification

Genomic DNA was extracted from cultured skin fibroblasts (Maniatis et al. 1982), and 1 μg of DNA was amplified under conditions essentially as reported for RT-PCR, using primers described in table 1. The PCR products were isolated by agarose gel electrophoresis and phenol extraction, for direct DNA sequencing.

Direct DNA Sequencing

Direct sequencing (Sanger et al. 1977) of the purified PCR products was performed using the double-stranded DNA cycle-sequencing system (BRL), by following the manufacturer's protocol. End-labeling of primers with T4 polynucleotide kinase (BRL) and γ-^{32}P-ATP (NEN) was performed as recommended by the supplier.

Allele-specific Oligonucleotide Hybridization

Amplified genomic DNA products were applied to a nylon filter, by using a Bio-Dot apparatus (BIO-RAD). The DNA was cross-linked by UV irradiation to the filter and baked at 80°C for 1 h. The filter was prehybridized briefly in 5 × SSPE, 0.5% SDS, and 5 × Denhardt's and then hybridized for 1 h at T_m −2°C (where T_m is the melting temperature of the probe, calculated as [4 × G+C content] + [2 × A+T content]) to probes specific to either normal or mutant alleles (table 1). The filters were washed twice briefly at room temperature in 2 × SSPE and 0.1% SDS and once for 10 min at the T_m of the probe and were autoradiographed at −70°C.

Inverse PCR

Our inverse PCR procedure is based on a method developed independently by Ochman et al. (1988), Triglia et al. (1988), and Silver and Keerikatte (1989). Multiple genomic DNA (1-μg) digests were performed, each using one of several restriction endonucleases (BRL) with 4-bp recognition sites, to generate small DNA fragments. After digestion, phenol extraction, and ethanol precipitation, the digest fragments at a final concentration of 4 μg/ml were ligated in a total volume of 20 μl containing buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and no polyethylene glycol) and 1 Weiss unit of T4 DNA ligase (BRL). Ligations were carried out at 15°C for 16 h. These conditions favor the formation of self-ligated circular DNA. Five-microliter samples were subjected directly to PCR amplification using primers (table 1) hybridizing to the exon of interest but with 3'OH termini oriented away from each other. Thus, only circularized DNA containing the exon supports the amplification and yields a product that can be identified on agarose gel.

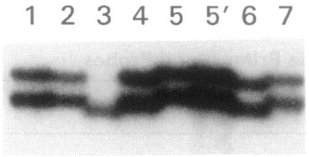

Figure 1 SSCP analysis of RT-PCR amplified DNA. The 555-bp product spanning all or part of exons 13–15 was digested with *Sph*I to generate fragments of 349 and 206 bp. Only the bands corresponding to the conformers of the 349-bp fragment are shown. Lane 1, Normal control. Lane 3, Patient 1. Lanes 5 and 5', Patient 2. Lane 7, Patient 3. Lanes 2, 4, and 6, PD patients reported elsewhere (disease control) whose amplified DNA product shows normal mobility.

Cell Labeling and Immunoprecipitation

Cell cultures were starved for 1 h in methionine-free medium (i.e., Eagle's MEM in 10 % FCS), labeled with 0.5 mCi Trans-^{35}S-L-methionine (>1000 Ci/mmol, NEN) for 3 hours at 37°C. The medium was removed, and the label was chased for 1 h in new medium supplemented with unlabeled methionine. Immunoprecipitation (Proia et al. 1984) was performed using a polyclonal rabbit antiserum (Boright et al. 1989). PAGE (Laemmli 1970) gels were autoradiographed using En^{3}Hance (Dupont). As a control for labeling and loading, ß-hexosaminidase was immunoprecipitated in each cell line (data not shown).

Results

SSCP Analysis

Aberrant band patterns indicative of mutations were observed in the *Sph*I digest fragments of an RT-PCR product that spans nucleotides 1085–1639 (containing exon 14 and parts of exons 13 and 15) in patients 1–3 (fig. 1, lanes 3, 5, 5', and 7). Differences in migration of the mutant fragments were more obvious in the digested DNA samples but could still be easily identified in the undigested DNA samples (data not shown). No electrophoretic mobility shifts were observed for any of the other RT-PCR products.

Direct Sequencing of Mutant cDNAs: (Patients 1–3)

Patient 1 had in exon 15 an in-frame 3-bp (GAG) deletion that results in the removal of glutamate residue 452 or 453 from the polypeptide. Neither SSCP analysis nor direct sequencing identified a second mRNA species in this patient. Amplification and sequencing of genomic DNA showed heterozygosity for the 3-bp de-

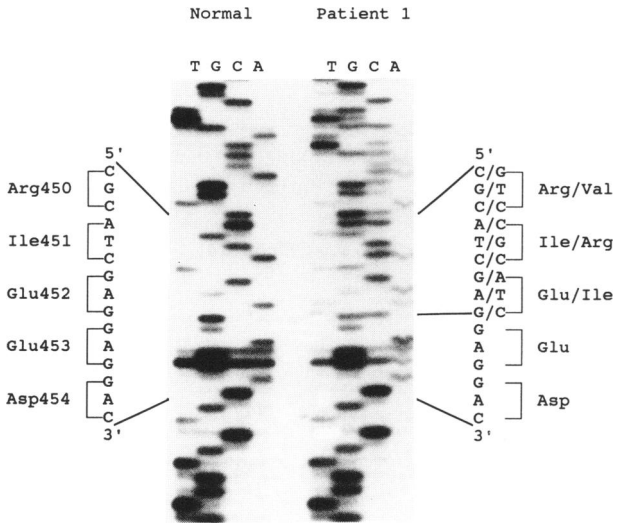

Figure 2 Patient 1, nucleotide sequence analysis of PCR-amplified genomic DNA. A double sequencing ladder appears at the Glu/Ile codon.

letion (fig. 2). The other allele was not identified and apparently is either expressed as a completely unstable mRNA or is not expressed at all. The 3-bp deletion was inherited from the mother.

Patients 2 and 3 carried a G→A transition at nt 1342 in exon 14, resulting in a G448R missense mutation. Neither patient's cDNA had normal band patterns on SSCP analysis. Genomic DNA spanning this region was amplified and sequenced. Patient 2 was homozygous for the mutation (fig. 3), while patient 3 was a compound heterozygote showing both the mutant and the normal base (fig. 3). The other allele in patient 3, presumably not expressed as mRNA, was not identified.

Allele-specific Oligonucleotide Hybridization: (Patients 1–3)

The 3-bp deletion (patient 1) and the G1342A substitution (patients 2 and 3) were confirmed. Neither mutation was found among 150 independently segregating normal chromosomes (data not shown).

Direct Sequencing of cDNAs with Deletions: (Patients 4 and 5)

RT-PCR amplification of a cDNA fragment spanning exons 4–11 revealed in patients 4 and 5 a single product shorter than normal. Direct sequencing of the mutant RT-PCR products demonstrated that exon 5 was absent in patient 4 (fig. 4) and that exon 7 was absent in patient 5 (fig. 5). These in-frame exon deletions result in the loss of 15 and 16 amino acid residues, respectively, from the polypeptide.

Inverse PCR and Sequencing of Unknown Flanking Introns: (Patients 4 and 5)

Inverse PCR was employed to identify the sequence of the intronic regions in normal genomic DNA. Inverse PCR of *Alu*I-digested and circularized genomic DNA yielded PCR products of ~500 bp and ~590 bp when exon 5 primers and exon 7 primers, respectively, were used. Digestion of products with *Alu*I confirmed the presence of a single restriction site. The two amplified products were directly sequenced, and their identities confirmed by comparison with known portions of the intronic sequences immediately following the primers (Tanoue et al. 1990*b*). Primers were designed (table 1) for conventional PCR of exons 5 and 7 and their intron borders from patients' genomic DNA. PCR products of the expected size were obtained (data not shown) and, on sequencing, revealed a G→C transversion at position −1 of intron 4 in one allele of patient 4 (fig. 6) and an A→G transition at position −2 of intron 6 in both alleles of patient 5 (fig. 7). Both mutations alter the invariant AG splice acceptor consensus sequence.

The intron 4 mutation (patient 4) removes an *Xba*I site and modifies the restriction-enzyme digestion pattern in the genomic PCR product (data not shown). The unidentified allele in patient 4 appears not to produce prolidase message, since RT-PCR amplification and cDNA sequencing detected the presence of only the exon 5–deleted cDNA.

Immunoreactivity of Mutant Skin Fibroblast Cell Lines

Metabolic labeling with ^{35}S-methionine, followed by immunoprecipitation of fibroblast prolidase subunit have been reported elsewhere for patients 1–4 (Boright et al. 1989). We analyzed the cultured fibroblasts of patient 5 in a similar way (fig. 8). Patient 2, (G→A, 1342/G→A, 1342) is cross-reacting material (CRM) positive; patient 3 (G→A, 1342/unknown) is CRM reduced. Patient 1 (3-bp deletion/unknown), patient 4 (IVS 4 -1/unknown), and patient 5 (IVS 6 -2/IVS 6 -2) are all CRM negative.

Discussion

We have identified four novel PEPD mutant alleles causing PD. As with other rare genetic disorders, heteroallelism—rather than the predominance of a single mutation—appears to account for the occurrence of

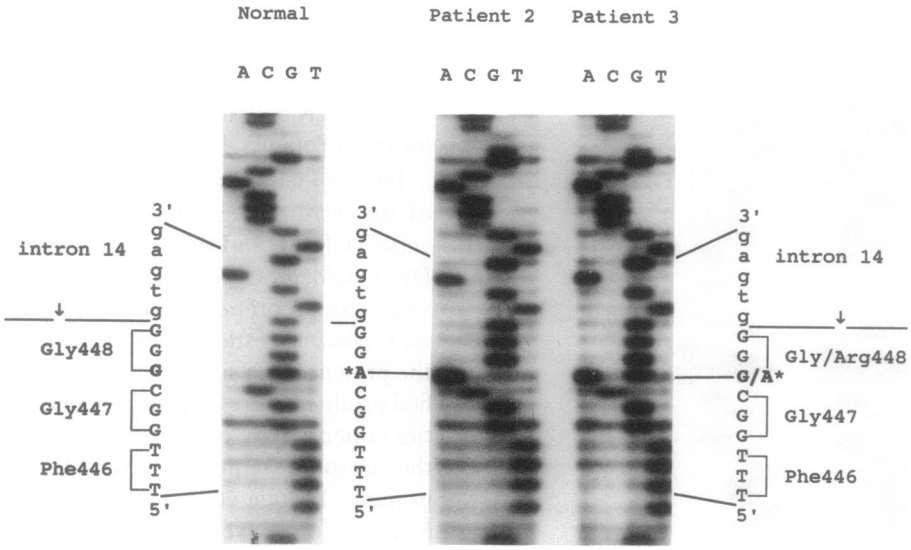

Figure 3 Patients 2 and 3, nucleotide sequence analysis of PCR-amplified genomic DNA. The asterisk (*) identifies the mutated nucleotide.

the disease. The PEPD alleles represent examples of well-known mutation mechanisms leading to loss of functional protein. The nonconservative amino acid substitution (G448R) occurs as a consequence of a mutation involving a CpG dinucleotide, a position that is known to be (because of spontaneous deamination of methylated cytosines) a hot spot for point mutations (Coulondre et al. 1978). The codon deletion (E452 or E453) occurs in a region of direct trinucleotide repeat and is presumed to arise by the mechanism of slipped-strand mispairing (Streisinger et al. 1966). Both mutations occur at conserved positions within a highly conserved amino acid sequence in a family of functionally related peptidases (Yoshimoto et al. 1989; Nakahigashi and Inokuchi 1990; Butler et al. 1993). These alleles do not appear to be polymorphisms, since neither was present in a sample of 150 independently segregating normal chromosomes.

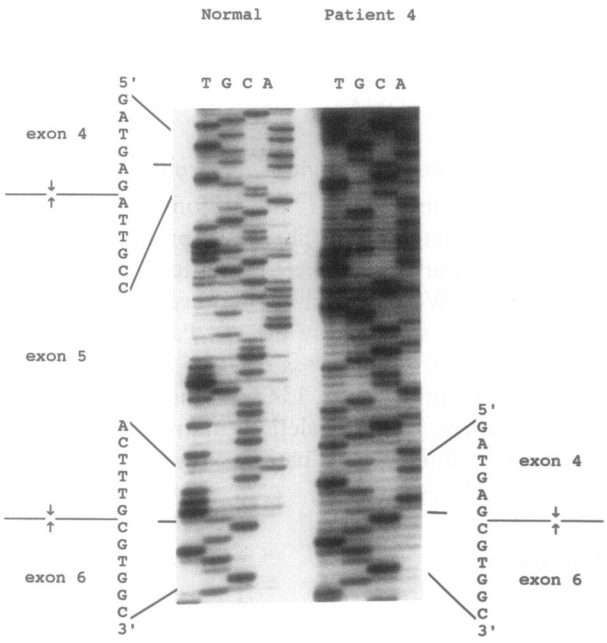

Figure 4 Patient 4. RT-PCR–amplified mRNAs were directly sequenced.

The two splicing defects disrupt the AG dinucleotide at the 3′ end of the intron that is absolutely necessary for correct splicing. Unlike some splice-site mutations, in which several abnormal mRNA species occur because of either preferential use of cryptic splice sites or deletion of multiple exons, the mutant alleles reported here produce a single messenger RNA species in which the exon following the affected splice signal is deleted in its entirety. The exon 5 and exon 7 in-frame deletions cause the loss of 15 and 16 amino acids, respectively, and presumably encode a protein whose stability and/or active site are profoundly affected.

In addition to the four mutant alleles described here, at least one and possibly three other mutations are present in these patients. Patients 1, 3, and 4 are compound

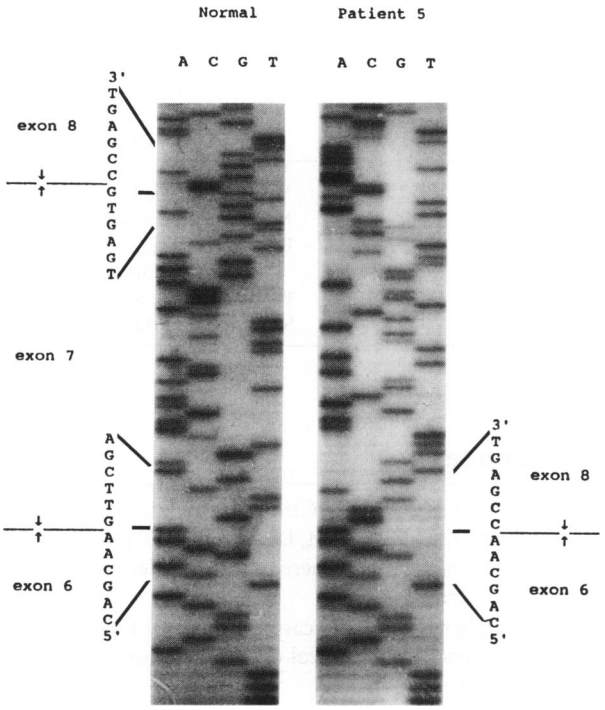

Figure 5 Patient 5. RT-PCR–amplified mRNA was directly sequenced.

heterozygotes with one identified and one unknown mutant allele that either does not synthesize prolidase mRNA or produces a very unstable transcript. Two other prolidase gene mutations—a 774-bp genomic DNA deletion that includes exon 14 (Tanoue et al. 1990*b*) and a G826A substitution in exon 12 (Tanoue et al. 1990*a*)—were ruled out in our patient samples, either on the basis of the size of the RT-PCR products (deletion) or by SSCP analysis (G826A substitution).

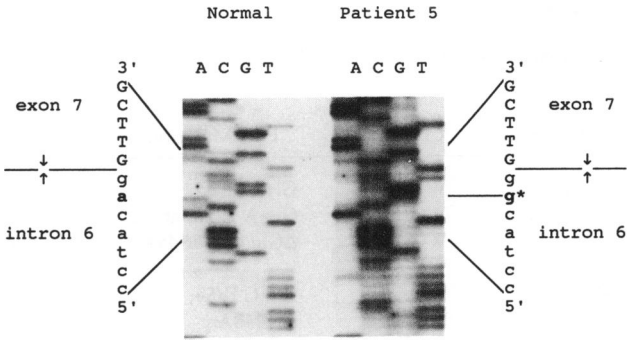

Figure 7 Patient 5, nucleotide sequence analysis of PCR-amplified genomic DNA. The asterisk (*) identifies the mutated nucleotide.

The clinical phenotype in PD is highly variable and includes some asymptomatic individuals with iminodipeptiduria and deficient prolidase who were ascertained through neonatal screening (Lemieux et al. 1984; Naughten et al. 1984). Functional assays based on erythrocyte prolidase activity or iminodipeptiduria do not predict clinical expression (Umemura 1978; Isemura et al. 1979; Gray et al. 1983; Lemieux et al. 1984; Naughten et al. 1984). Ultimately the prediction of phenotype may depend on a combination of both functional assays and comparison of mutant alleles in each family with a catalog of published mutations whose phenotypes have been investigated. The genotype-

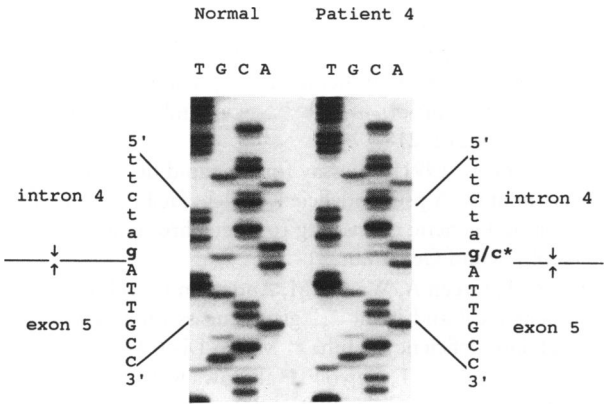

Figure 6 Patient 4, genomic DNA nucleotide sequence analysis of PCR-amplified genomic DNA. The asterisk (*) identifies the mutated nucleotide.

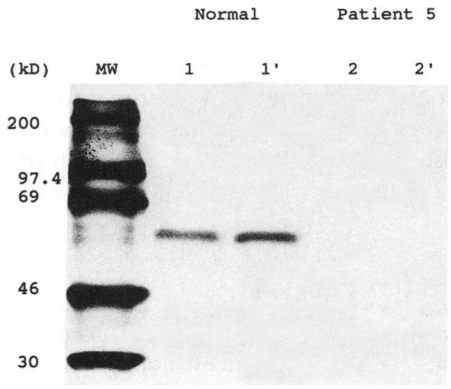

Figure 8 Immunoprecipitation of the prolidase subunit. SDS-PAGE was performed on a 12% gel. Lanes 1 and 1′, Normal control. Lanes 2 and 2′, Patient 5.

Table 2

Phenotypes and Genotypes of Prolidase-deficient Patients

Patient No.	Cell Line	First Mutation	Second Mutation	Age at Onset (years)	Clinical Phenotype[a]
3	WG 1343	Gly448Arg	Null allele	0–2	Mild MR, RI, mild skin lesions
5	WG 1625	G→A IVS6; nt −2	G→A IVS6; nt −2	0–2	Mild MR, skin ulcers
1	WG 1082	ΔGlu 452 or 453	Null allele	0–2	Borderline MR, mild skin lesions, chronic liver disease
4	WG 1298	G→C IVS4; nt −1	Null allele	2–10	Borderline MR, RI, skin ulcers
2	WG 1194	Gly448Arg	Gly448Arg	10–20	Skin ulcers, no MR

[a] MR = mental retardation; and RI = recurrent infections.

phenotype correlations for the patients investigated here are presented in table 2.

The two splice-site mutations and the codon deletion reported here, whether in the homozygous state or in combination with alleles not expressed as mRNA, are all associated with the severe form of PD (patients 1, 4, and 5). The G448R allele produces a severe phenotype in combination with a null allele (patient 3, age at onset <2 years) but causes a much milder phenotype when in the homozygous state (patient 2 and her brother, age at onset >13 years), despite the small differences in residual enzymatic activities detected in cultured fibroblasts from the two patients (homozygote 9.4% of control value and compound heterozygote 13.1% of control value, respectively; Boright et al. 1989).

It is hoped that the characterization of PEPD mutations may lead to predictive tests that distinguish severe from mild or asymptomatic cases. At present, allele-specific tests can distinguish between the pseudodeficient allele at the ARSA locus and alleles causing metachromatic leukodystrophy (Gieselmann 1991; Francis et al. 1993) and can distinguish between alleles causing either benign hexosaminidase A deficiency (Triggs-Raine et al. 1992) and adult or chronic forms of Tay-Sachs disease (Navon and Proia 1989; Paw et al. 1989) and those causing the infantile-onset form of the disease.

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