Am. J. Hum. Genet. 59:1035–1039, 1996

Expression and Molecular Analysis of Mutations in Prolidase Deficiency

Pierre Ledoux,[1,2] Charles R. Scriver,[1,2,3] and Peter Hechtman[1,2,3]

[1]Division of Medical Genetics, DeBelle Laboratory, Montreal Children's Hospital Research Institute, and Departments of [2]Biology and [3]Human Genetics, McGill University, Montreal

Summary

Prolidase (E.C.3.4.13.9) cleaves iminodipeptides. Prolidase deficiency (PD; McKusick 170100) is an autosomal recessive disorder with highly variable penetrance. We have identified two novel alleles in the prolidase gene (PEPD) by direct sequencing of PCR-amplified cDNA from a PD individual asymptomatic at age 11 years: a 551G→A transition in exon 8 (R184Q) and a 833G→A transition in exon 12 (G278D). To assess the biochemical phenotypes of these and two previously identified PEPD mutations (G448R and delE452), we have designed a transient-expression system for prolidase in COS-1 cells. The enzyme was expressed as a fusion protein carrying an N-terminal tag, the HA1 epitope of influenza hemagglutinin, allowing its immunological discrimination from the endogenous enzyme with a monoclonal antibody. Expression of the R184Q mutation produced 7.4% of control enzymatic activity whereas the expression of the G278D, G448R, and delE452 mutations produced inactive enzymes. Western analysis of the R184Q, G278D, and G448R prolidases revealed stable immunoreactive material whereas the delE452 prolidase was not detectable. Pulse-chase metabolic labeling of cells followed by immunoprecipitation revealed that the delE452 mutant protein was synthesized but had an increased rate of degradation.

Introduction

Prolidase is an enzyme that hydrolyzes dipeptides with carboxy-terminal proline or hydroxyproline. It is a homodimeric, cytosolic, ubiquitous enzyme with a specific requirement for manganese (Davis and Smith 1957; Sjöström et al. 1973). Prolidase plays a major role in the recycling of proline formed during the final stages of degradation of collagen and dietary proteins (Jackson et al. 1975; Powell and Maniscalco 1976). Prolidase deficiency (PD) is a rare autosomal recessive disorder with highly variable expressivity. The phenotype most frequently involves chronic skin ulcers, mental retardation, and recurrent infections. The age at onset of the disease varies, from birth to 22 years of age, and some cases are asymptomatic (Phang et al. 1995). The prolidase gene (PEPD), contains 15 exons spanning >130 kb of DNA (Tanoue et al. 1990) and is located on chromosome 19p13.2, and the 2.2-kb mRNA encodes a polypeptide of 493 amino acids (Endo et al. 1989). Elucidation of the cDNA sequence has allowed the search for mutations in PD individuals. Elsewhere we have described four mutant PEPD alleles associated with the disease (Ledoux et al. 1994), and two novel alleles are reported here. We have developed a novel expression system to study mutant PEPD alleles by using COS-1 cells and have determined that four of these mutations are responsible for the enzyme deficiency. Furthermore, we have determined which of two mutant alleles in an asymptomatic compound heterozygote is benign.

Received June 3, 1996; accepted for publication August 27, 1996.

Address for correspondence and reprints: Dr. Peter Hechtman, Division of Medical Genetics, DeBelle Laboratory, Montreal Children's Hospital Research Institute, 2300 Tupper Street, Montreal, Quebec H3H 1P3, Canada. E-mail: hechtman@bio1.lan.mcgill.ca

0002-9297/96/5905-0011$02.00

Material and Methods

Cell Strains

Cultured fibroblasts and COS-1 cells were grown in Eagle's minimal essential medium (BRL) supplemented with 10% FCS and Dulbecco's minimal essential medium (BRL) containing 10% FBS, respectively. Biochemical, phenotypic, and genotypic data on the cell lines described in this study have been reported elsewhere (Jackson et al. 1975; Gray et al. 1983; Boright et al. 1989; Ledoux et al. 1994). Mutation analysis was performed on strain WG1077 from the Repository of Mutant Cell Strains, Montreal Children's Hospital.

Reverse Transcription–PCR Amplification and Direct Sequencing

RNA was isolated and reverse transcribed, and the entire coding region of the prolidase cDNA was amplified as four overlapping fragments, as described elsewhere (Ledoux et al. 1994). The PCR products were gel purified and directly sequenced by use of the dsDNA cycle sequencing system (BRL).

Construction of the Expression Vector

Nucleotides 1-1591 of the prolidase cDNA were PCR amplified with primers carrying 5′ overhang sequences containing restriction sites immediately adjacent to the cDNA and were subcloned into plasmid BluescriptII (Stratagene). The resulting plasmid was digested with *Xba*I and *Bam*HI, and the insert was subcloned into the expression vector pSVL (Pharmacia) to create pFX3. A cassette encoding a triple repeat of the HA1 epitope of hemagglutinin of the influenza virus, carrying an ATG start codon, was ligated into linearized pFX3. This vector had been digested with *Xba*I, to cut immediately upstream of the start codon, and treated with mung-bean nuclease, to remove the overhang. The resulting plasmid pPROL contained the entire coding region of prolidase and 109 bases of the 3′ UTR, in frame with a cassette encoding the triplicated HA1 epitope on the 5′ side.

Mutagenesis

The transformer site–directed mutagenesis kit from Clontech was used to create the mutant expression vectors. The pFX3 vector served as the wild-type DNA template. Mutagenic primers of 28–33 bp were used to introduce the following mutations: 551G→A (R184Q), 833G→A (G278D), 1342G→A (G448R), and delGAG1354-1356 (delE452). Mutant expression vectors were created by substituting short restriction fragments from pPROL with the corresponding mutagenized fragments. The nucleotide sequence and orientation of the substituted fragments were confirmed by sequencing. Each plasmid was purified by two rounds of CsCl/ethidium bromide centrifugation.

Transient Expression of HA1-Prolidase in COS-1 Cells

COS-1 cells were harvested when they reached ~60% confluence. Approximately 10^7 cells were transfected with 10 μg of either wild-type or mutant prolidase expression vector in 0.5 ml of medium. Two micrograms of the plasmid pCMVβGal, which harbors the β-galactosidase gene of *Escherichia coli,* was cotransfected to standardize for transfection efficiency. A mock transfectant was prepared by transfection with pSVL and pCMVβ-Gal. Electroporation was performed with a BioRad GenePulser set at infinite capacitance, 280 V, and 960 μF. Cells were harvested 64 h posttransfection. Cells were resuspended in 0.25 M Tris pH 7.8, and lysates were prepared by three rounds of freezing and thawing. Protein concentration was measured by use of the Folin-Ciocalteu reagent. β-Galactosidase activity was assayed by use of a fluorogenic substrate (Sambrook et al. 1989).

Prolidase Assay

Prolidase activity was measured as by Myara et al. (1982). Transfected enzymatic activity was distinguished from endogenous COS activity by immunoprecipitation with a monoclonal antibody against influenza hemagglutinin. Cell extracts containing 20 μg of protein were preincubated 16 h in preincubation buffer (50 mM Tris pH 7.8 and 1 mM $MnCl_2$) at 37°C, the standard method of activation of prolidase. One microliter of raw ascites fluid containing the monoclonal antibody 12CA5 (Babco) raised against the HA1 epitope was added, and extracts incubated at 4°C for 1 h. Twenty-five microliters of *Staphylococcus aureus* protein A centrifuged and resuspended in the same volume of preincubation buffer were added, and extracts were incubated at 4°C for 1 h. The mixture was centrifuged at 15,000 *g* for 20 s. The pellet was washed twice with 50 mM Tris pH 7.8 and was resuspended in preincubation buffer. Prolidase activity in resuspended pellets was assayed as above. One unit of prolidase activity is defined as the amount of enzyme that releases 1 μmol of proline/h under standard conditions.

Metabolic Labeling

Metabolic labeling of transfected COS-1 cells was performed 64 h after transfection in 75-cm^2 tissue-culture flasks. Cells were starved for 1 h in methionine-free medium and then were labeled for 3 h with 0.5 mCi Trans-[^{35}S]-L-methionine (>1,000 Ci/mmol; NEN). In some cases, a 5-h chase was carried out by replacing the medium with new medium supplemented with unlabeled methionine. Immunoprecipitation of cell lysates with the anti-HA1 antibody was performed according to the method of Proia et al. (1984).

Western Analysis

Fifty micrograms of protein from transfected cell lysates were separated by 12% SDS-PAGE and were transferred to nitrocellulose membranes (Hybond-C extra; Amersham). Prolidase was detected by use of a 1:5,000 dilution of anti-HA1 (primary) antibody and the ECL Western Blotting kit (Amersham), according to the manufacturer's protocol.

Results

Identification of Mutations

Mutation analysis was performed on the cultured skin-fibroblast cell line of an asymptomatic PD individual with iminodipeptiduria. Direct sequencing revealed two nucleotide substitutions: a 551G→A transition in exon 8, resulting in an R184Q substitution (fig. 1), and a 833G→A transition in exon 12, resulting in a G278D substitution (fig. 2). The presence, on sequencing gels, of both the normal and the mutated base at positions 551 and 833 of the cDNA indicated that the proband is a genetic compound for two mutant alleles on homologous chromosomes. No other alterations were found

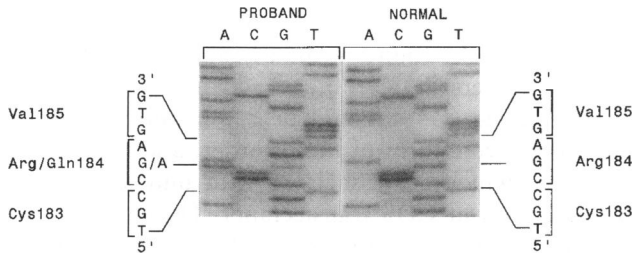

Figure 1 Nucleotide sequence analysis of normal and mutant PCR-amplified cDNA. The patient is heterozygous for the 551G→A allele (R184Q).

by sequencing the complete coding region. Direct sequencing of amplified parental cDNAs showed that the 551G→A allele was paternal and that the 833G→A allele was maternal (data not shown).

Prolidase Expression in COS-1 Cells

The effect that the R184Q, and G278D, and G448R substitutions and the E452 deletion had on prolidase was measured by expression analysis in COS-1 cells. After transfection of COS-1 cells with the HA1-prolidase fusion gene, prolidase activity in the cell lysates was 18.7 and 53.7 units/mg for mock and wild-type transfectants, respectively. The difference between the two activities, corresponding to the activity of the enzyme expressed from the transfected wild-type cDNA, was twofold greater than the endogenous activity. After immunoprecipitation with anti-HA1 antibody, 88% of the human prolidase activity expressed in wild-type transfectants (30.8 units/mg) was recovered in the immunoprecipitates (fig. 3). Immunoprecipitable activity in mock transfected cells was <0.48 units/mg. Recovery of human prolidase activity was not improved by eluting the enzyme from the immunoprecipitates with the HA1 peptide.

The R184Q mutant enzyme had 7.4% of wild-type activity (2.3 units/mg) (fig. 3). No enzyme activity was detected in cells transfected with the G278D, G448R, and the delE452 mutant cDNAs (fig. 3). Cotransfection with a reporter gene produced β-galactosidase activities

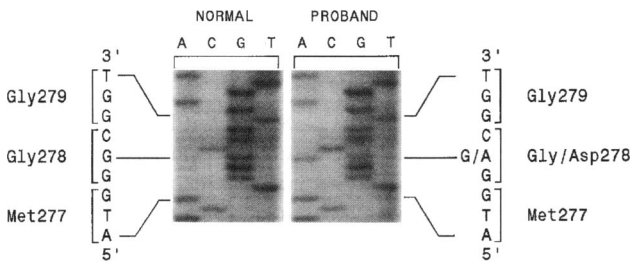

Figure 2 Nucleotide sequence analysis of normal and mutant PCR-amplified cDNA. The patient is heterozygous for the 833G→A allele (G278D).

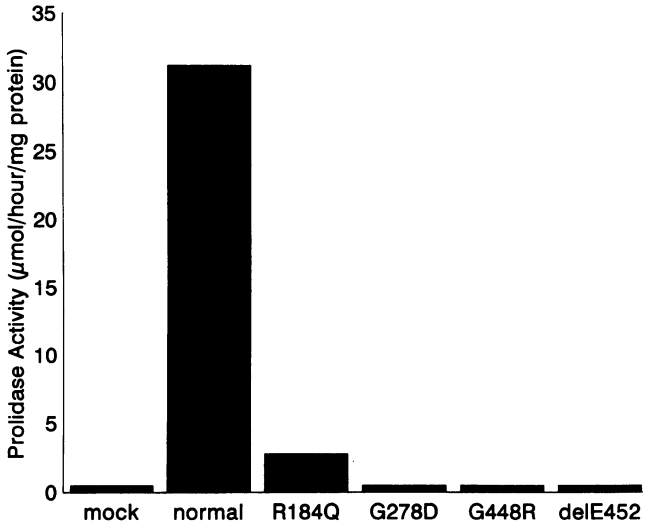

Figure 3 Expression of wild-type and mutant PEPD alleles in COS-1 cells. Prolidase activity was measured on resuspended pellets after immunoprecipitation with anti-HA1 antibody.

in the wild-type and mutant transfectants that were 93%–100% of that found in the mock transfect. Because of the narrow variation, prolidase-activity values were not adjusted.

We investigated expression of normal and mutant cDNAs in transfected cell extracts by western analysis using anti-HA1 antibody (fig. 4). A normal amount of immunoreactive protein, of the expected size (60 kD), was detected in COS-1 cells transfected with wild-type or mutant cDNAs for R184Q, G278D, and G448R alleles. Cells transfected with cDNA carrying the delE452 mutation produced undetectable or barely detectable amounts of immunoreactive protein. Pulse-chase studies suggested that the delE452 protein was synthesized but unstable. The level of mutant protein after a 5-h chase was drastically reduced. The level of wild-type protein remained unchanged during the 5-h chase (fig. 5).

Discussion

Neonatal screening for PD has identified asymptomatic individuals with the metabolic features of the disor-

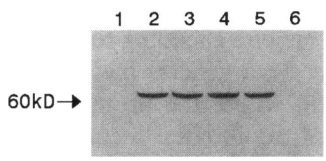

Figure 4 Western blot analysis of COS-1 cells expressing the 60-kD HA1-prolidase fusion polypeptide. An antibody directed against the HA1 epitope was used. Fifty micrograms of protein from extracts of mock-transfected cells (lane 1) and from cells transfected with vectors harboring the wild-type cDNA (lane 2), the R184Q allele (lane 3), the G278D allele (lane 4), the G448R allele (lane 5), and the delE452 allele (lane 6) were loaded onto a 12% polyacrylamide gel.

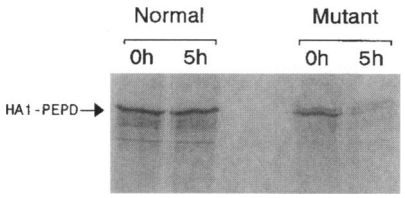

Figure 5 SDS-PAGE of immunoprecipitated HA1-prolidase from labeled COS-1 cells transfected with normal and delE452 PEPD cDNAs. Cells were labeled for 3 h and then were chased for either 0 or 5 h. Cells were then lysed, and the expressed HA1-prolidase fusion polypeptide was immunoprecipitated with an anti-HA1 antibody.

der (Lemieux et al. 1984; Naughten et al. 1984). Since prolidase-deficient sibs with different ages at onset have been reported (Umemura 1978; Isemura et al. 1979), it is not certain how long these individuals will remain symptom free. Accordingly, we have developed a system to express human PEPD alleles in COS cells, in order to assess the contribution of PEPD mutations to the occurrence of the clinical disorder.

The principal difficulty in expressing mutant human genes encoding "housekeeping" functions is that the host cell expresses the same enzymes, thereby preventing accurate estimation of low levels of residual enzymatic activity that are associated with the expression of mutant human genes. Therefore, we constructed an expression vector encoding a chimeric protein that incorporated the influenza hemagglutinin epitope at the N-terminus of the human prolidase polypeptide. The choice of epitope was dictated by the commercial availability of a highly specific monoclonal antibody directed against influenza hemagglutinin.

Human prolidase expressed from the pSVL vector produced enzymatic activity threefold greater than that in mock-transfected COS cells. Western analysis of wild-type transfectants, using anti human prolidase as primary antibody, detected both endogenous (COS enzyme) and chimeric (expressed) prolidase, producing band intensities that were proportional to enzymatic activity (data not shown), indicating that the N-terminal epitope does not interfere with the catalytic function of the enzyme. Recovery of human enzyme after anti-HA antibody immunoprecipitation was virtually complete. Addition of a cassette encoding the HA1 epitope to any gene thus may be a general method for heterologous gene expression where the protein product is not required to cross the internal membrane systems of the cell.

The asymptomatic proband previously had been shown to have 8.2% of normal prolidase activity in cultured fibroblasts (Boright et al 1989). A severely affected patient carrying PEPD null alleles (WG1298) also showed a similar level of residual fibroblast prolidase activity, indicating that low levels of fibroblast prolidase do not reliably predict clinical outcome. In our studies, one of the alleles found in the asymptomatic proband, R184Q, confers 7.4% residual activity, in contrast to the two alleles G448R and delE452, both known to cause the severe form of the disorder, and the G278D mutation, which are associated with undetectable enzyme activity in COS transfectants. The more precise genotype-phenotype correlation, obtained by measurement of immunoprecipitated human prolidase expressed in COS cells, is likely to be related to the presence, in human fibroblasts, of variable amounts of prolidase II (Butterworth and Priestman 1985), an unrelated imidodipeptidase that does not appear to modify the clinical phenotype.

The putative active site of prolidase is formed by six core β-strands (Bazan et al. 1994; Mock and Liu 1995). Five highly conserved residues within these β-motifs bind active-site metal ions. The G278D and G448R mutations occur within these β-motifs, and both introduce charged side chains in close proximity to—and on the same surface as—the negatively charged metal-binding residues D276 and E452. These substitutions do not affect the stability of the catalytically inactive enzyme. We speculate that steric and/or electronic hindrance affecting the function of the metal-binding residues might explain the prolidase-activity loss associated with these mutations. The delE452 mutation removes one of the metal-coordinating residues; however, it is likely that the destabilizing effect of this mutation is due to the reversal of polarity in the distribution of residues within the β-motif proximal to the mutation.

The mutation R184Q occurs in a nonconserved region of the protein, distal from the active site. Therefore, it is not surprising that the enzyme remains partially active. These studies provide further evidence for the validity of the current structural model of prolidase.

Acknowledgments

The authors thank the Medical Research Council of Canada for supporting this research. P.L. also acknowledges the support of FCAR, the Montreal Children's Hospital Research Institute, and the McGill Medical Faculty.

References

Bazan JF, Weaver LH, Roderick SL, Huber R, Matthews BW (1994) Sequence and structure comparison suggest that methionine aminopeptidase, prolidase, aminopeptidase P, and creatinase share a common fold. Proc Natl Acad Sci USA 91:2473–2477

Boright AP, Scriver CR, Lancaster GA, Choy F (1989) Prolidase deficiency: biochemical classification of alleles. Am J Hum Genet 44:731–740

Butterworth J, Priestman DA (1985) Presence in human cells

and tissues of two prolidases and their alteration in prolidase deficiency. J Inherit Metab Dis 8:193–197

Davis NC, Smith EL (1957) Purification and some properties of prolidase of swine kidney. J Biol Chem 224:261–275

Endo F, Tanoue A, Nakai H, Hata A, Indo Y, Titani K, Matsuda I (1989) Primary structure and gene localization of human prolidase. J Biol Chem 264:4476–4481

Gray RGF, Green A, Ward AM, Anderson I, Peck DS (1983) Biochemical and immunological studies on a family with prolidase deficiency. J Inherit Metab Dis 6 Suppl 2:143–144

Isemura M, Hanyu T, Gejyo F, Nakazawa R, Igarashi R, Matsuo S, Ikeda K, et al (1979) Prolidase deficiency with imidodipeptiduria: a familial case with and without clinical symptoms. Clin Chim Acta 93:401–407

Jackson SH, Dennis AW, Greenberg MB (1975) Iminodipeptiduria: a genetic defect in recycling collagen: a method for determining prolidase in erythrocytes. Can Med Assoc J 113:759–763

Ledoux P, Scriver C, Hechtman P (1994) Four novel PEPD alleles causing prolidase deficiency. Am J Hum Genet 54: 1014–1021

Lemieux B, Auray-Blais C, Giguere R, Shapcott D (1984) Prolidase deficiency: detection of cases by a newborn urinary screening programme. J Inherit Metab Dis 7 Suppl 2:145–146

Mock WL, Liu Y (1995) Hydrolysis of picolinylprolines by prolidase. J Biol Chem 270:18437–18446

Myara I, Charpentier C, Lemonnier A (1982) Optimal conditions for prolidase assay by proline colorimetric determination: application to iminodipeptiduria. Clin Chim Acta 125: 193–205

Naughten ER, Proctor SP, Levy HL, Coulombe JT, Ampola MG (1984) Congenital expression of prolidase defect in prolidase deficiency. Pediatr Res 18:259–261

Phang JM, Yeh JC, Scriver CR (1995) Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 7th ed. McGraw-Hill, New York, pp 1125–1146

Powell GF, Maniscalco RM (1976) Bound hydroxyproline excretion following gelatin loading in prolidase deficiency. Metabolism 25:503–508

Proia RL, d'Azzo A, Neufeld EF (1984) Association of α and β-subunits during the biosynthesis of β-hexosaminidase in cultured human fibroblasts. J Biol Chem 259:3350–3354

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sjöström H, Noren O, Josefsson L (1973) Purification and specificity of pig intestinal prolidase. Biochim Biophys Acta 327:457–470

Tanoue A, Endo F, Matsuda I (1990) Structural organization of the gene for human prolidase (petidase D) and demonstration of a partial gene deletion in a patient with prolidase deficiency. J Biol Chem 265:11306–11311

Umemura S (1978) Studies on a patient with imidodipeptiduria. II. Lack of prolidase activity in blood cells. Physiol Chem Phys Med 10:279–283