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Research Article

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Molecular Defect in Siblings with Prolidase Deficiency and Absence or Presence of Clinical Symptoms

A 0.8-kb Deletion with Breakpoints at the Short, Direct Repeat in the PEPD Gene and Synthesis of Abnormal Messenger RNA and Inactive Polypeptide

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Abstract

Prolidase deficiency is an autosomal recessive disorder with highly variable symptoms, including mental retardation, skin lesions, and abnormalities of collagenous tissues. In Japanese female siblings with polypeptide negative prolidase deficiency, and with different degrees of severity of skin lesions, we noted an abnormal mRNA with skipping of 192 bp sequence corresponding to exon 14 in lymphoblastoid cells taken from these patients. Transfection and expression analyses using the mutant prolidase cDNA revealed that a mutant protein translated from the abnormal mRNA had an M_r of 49,000 and was enzymatically inactive. A 774-bp deletion, including exon 14 was noted in the prolidase gene. The deletion had termini within short, direct repeats ranging in size of 7 bp (CCACCCT). The "slipped mispairing" mechanism may predominate in the generation of the deletion at this locus. This mutation caused a 192-bp in-frame deletion of prolidase mRNA and was inherited from the consanguineous parents. The same mutation caused a different degree of clinical phenotype of prolidase deficiency in this family, therefore factor(s) not related to the PEPD gene product also contribute to development of the clinical symptoms. Identification of mutations in the PEPD gene from subjects with prolidase deficiency provides further insight into the physiological role and structure-function relationship of this biologically important enzyme. (*J. Clin. Invest.* 1991. 87:1171–1176.) Key words: peptidase D • polymerase chain reaction • mutation • slipped mispairing • transfection

Introduction

Prolidase (peptidase D, EC 3, 4, 13, 9), which releases carboxy-terminal proline or hydroxyproline from oligopeptides, is an ubiquitous oligopeptidase present in mammals.

Human prolidase, a polymorphic protein (1) is a genetic marker on chromosome 19. A tight linkage between the prolidase gene (PEPD gene) and the myotonic dystrophy gene has been established (2). We reported that human prolidase is a homo-dimer with a subunit of M_r 54,300 and that the PEPD gene is on the short arm of chromosome 19 (3), spans over 130 kb and consists of 15 exons (4). The mature subunit of the protein is composed of 492 amino acid residues and candidates of amino acid substitutions for polymorphisms have been proposed (3, 4).

Prolidase deficiency is a syndrome characterized by skin lesions, abnormalities of bone and tendorious tissues, frequent infections, and mental retardation (5). These symptoms are caused by heterogeneous abnormalities of the PEPD gene product (6). The clinical symptoms of prolidase deficiency are variable and the severity of the disease does not seem to be related to factors linked to the enzymatic defects. An analysis of prolidase deficiency at the level of protein or mRNA led to speculation that the mRNA-positive patients are not mentally retarded (7). However, the severity of other symptoms, in particular the skin lesions could not be explained by the presence or absence of immunologically cross-reacting material (CRM) or mRNA in cells from these patients (7). Different skin lesions were evident among members of the same family. We have now analyzed molecular events in one such family in an attempt to determine whether there differences existed in gene mutations among the affected subjects in one family with skin lesions of different degrees of severity.

Methods

Northern blot analysis. Total cellular RNAs from lymphoblastoid cells from the control and patients were prepared using the guanidium thiocyanate procedure (8). Poly $(A)^+$ RNA from control human placenta was further purified on an oligo-(*dT*)-cellulose column. RNAs were separated on a 1.0% formaldehyde agarose gel, transferred to a nitrocellulose filter, and hybridized to prolidase cDNA insert (3). The DNA fragment was radiolabeled with [α-^{32}P] dCTP (3,000 Ci/mmol) (ICN Radiochemical, Irvine, CA) by multiple priming (9).

Southern blot analysis. Human genomic DNA was prepared from lymphoblastoid cells obtained from patients with prolidase deficiency and peripheral leukocytes from the control and the patient's parents. The DNA were digested with restriction enzymes, subjected to agarose gel electrophoresis, and the digested DNA was blotted onto nitrocellulose filters and hybridized to probes, as described (4). Probes were derived from prolidase cDNA inserts or from genomic phage clones (4).

Polymerase chain reaction (PCR) of mRNA and genomic DNA. A specific first-strand cDNA copy of human prolidase RNA was prepared from 10 μg of total cellular RNA and PCR of the cDNA was performed, as described (10). For the PCR of genomic DNA, ~ 1 μg of DNA was amplified with the use of a set of oligonucleotide primers complementary to the normal sequence of the human prolidase gene (4). Amplification of the cDNA or genomic DNA was carried out using Taq DNA polymerase (Takara Shuzo Co., Tokyo, Japan). 30 cycles of PCR amplification were performed with a DNA thermal cycler (Perkin-Elmer Cetus Corp., Hayward, CA). Each cycle was allowed to run for 1 min at 94°C to denature the double-stranded DNA, 2 min at 55°C for the primers to anneal the complementary sequence, and 5 min at 72°C for

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extension of the DNA strands. 10 μl of each 100 μl of PCR reaction mixture was analyzed on a 0.8% agarose gel, followed by staining with ethidium bromide. The DNAs in the gel were transferred to nitrocellulose filters and confirmed by Southern blot analyses using the prolidase cDNA insert as a probe. Specific amplified DNA fragments of the human prolidase were ligated into the plasmid vector pUC18 with T4 DNA ligase (Takara Shuzo Co.). Several independent clones obtained at each PCR amplification procedure were sequenced according to the dideoxy-chain termination method (11) using synthetic oligonucleotide primers complementary to the sequence of the vector, the prolidase cDNA or the prolidase gene.

Plasmid construction. A wild type of an expression clone (pEPD-W) was constructed as described (10): The 1.7-kb Eco RI fragment derived from prolidase cDNA was isolated and inserted into an expression vector pCAGGS, a derivative of pAGS-3 (12) and which has the cytomegalovirus intermediate early enhancer, the chicken β-actin promoter, and a polyadenylation signal of the rabbit β-globin gene.

A mutant type of an expression clone (pEPD-K) was produced by substitution the 938-bp Sma I fragment (698–1636) of human prolidase cDNA insert in pEPD-W with a 746-bp Sma I fragment with a 192-bp deletion of exon 14 derived from a subclone of the patient's cDNA. The nucleotide sequence and a proper 5′ to 3′ orientation of the prolidase cDNA in each clone was confirmed by restriction enzyme digestion, gel analysis, and sequence. Plasmids were grown in *Escherichia coli* HB101, then were purified by two cycles of cesium chloride–ethidium bromide centrifugation.

Preparation of cell extract and DNA transfection. BMT-10 cells derived from a monkey cell line (13) and used for the transfection were grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories) and maintained in a 5% CO_2 atmosphere at 37°C. Transfection of DNA was performed using lipofection (Bethesda Research Laboratories, Gaithersburg, MD) (14), according to instructions from the supplier. Nearly confluent cells (80%) in 75-cm dishes were washed twice with 6 ml of Opti MEM medium (Gibco Laboratories) and 18 ml of Opti MEM medium containing 10 μg of DNA and 30 μg of lipofection was added. To normalize for any variability in the transfection efficiency, plasmid pCH110, which contained the *E. coli* lac Z gene sequence under the control of simian virus 40 (SV 40) enhancer-promoter (15), was co-transfected. Cells were incubated for 24 h in a 7% CO_2 atmosphere at 37°C, after which the medium was replaced with 18 ml of RPMI 1640 medium with 10% FCS and the cells were harvested 2 d later. Cell pellets were suspended in ice-cold PBS, disrupted by sonication, then centrifuged at 12,000 *g* for 3 min. The cell extracts were used for protein assay, prolidase assay, immune precipitation, and immune blot analyses. Part of the cell extracts were assayed for β-galactosidase activity by incubation with ONPG (*O*-nitrophenyl-β-D-galactopyranoside), as described (16). The amount of protein was measured using a dye binding method (kit from Bio-Rad Laboratories, Richmond, CA).

Enzyme assay, immune precipitation, and immune blot. Purified human prolidase, monoclonal antibodies (EP2, EP10, and EP38) directed to human prolidase and antiserum raised against human prolidase were prepared as described (17). Prolidase activities in the crude cell extracts were measured as described (18). Immune precipitation of human prolidase with the mouse monoclonal antibody (EP2, EP10, or EP38) was carried out as described (17). The prolidase activity in the immune complex was measured as described (7) and conditions for immune blots were also as described (17).

Patients. Cultured lymphoblastoid cells were obtained from two Japanese female siblings with prolidase deficiency. Clinical features of these patients have been described elsewhere (19). These children were products of consanguineous parents. The older sister, now 25 yr of age developed skin lesions at the age of 19 mo and specific treatment was required. Her sister had no prominent change in the skin until age 18 yr. Both were negative for immunologically cross-reacting material and there were no residual activities of prolidase in the fibroblasts (7). Urine analysis for amino acids and oligopeptides revealed that the siblings excreted massive amounts of imidodipeptide (19). In the parents who were first cousins, erythrocyte prolidase activities were ~ 50% of the control value (20).

Results

In the skin fibroblasts taken from the siblings then cultured, there was no catalytic activity or immunological protein related to prolidase. Essentially the same results were obtained with cultured lymphoblastoid cells of which the immunological protein of 56 kD was absent (Fig. 1 *A*). The results of Northern blot analysis indicated that a 2.1-kb species of mRNA was present in the patients' cells, whereas the normal cells carried a 2.3-kb mRNA (Fig. 1 *B*). We analyzed the structure of mRNA using PCR techniques (Fig. 2 *A*). Nucleotide sequencing of the PCR-amplified cDNA from the patients revealed that 192 nucleotides corresponding to exon 14 were absent (Fig. 2 *B*). The deletion in mRNA did not disrupt the frame, and 64 amino acid residues were eliminated from the predicted protein sequence (Fig. 2 *B*). This is the only mutation we detected in these patients.

An analysis of transfection and expression was made to investigate the nature of the mutant protein translated from the abnormal mRNA. We constructed an expression clone pEPD-W, with a normal sequence of human prolidase and a pEPD-K with the 192-bp–deletion mutation. Immune blot analysis of the cell extracts from the transfection experiments indicated that an immunological protein of 56 kD was present in the cells transfected with pEPD-W (Fig. 3 *A*, lane *3*) whereas a smaller size of protein (49 kD) was present in cells transfected with pEPD-K (Fig. 3 *A*, lane *2*). Small amounts of endogenous monkey prolidase from BMT-10 cells were present in cells transfected with the expression vector (pCAGGS) alone (Fig. 3 *A*,

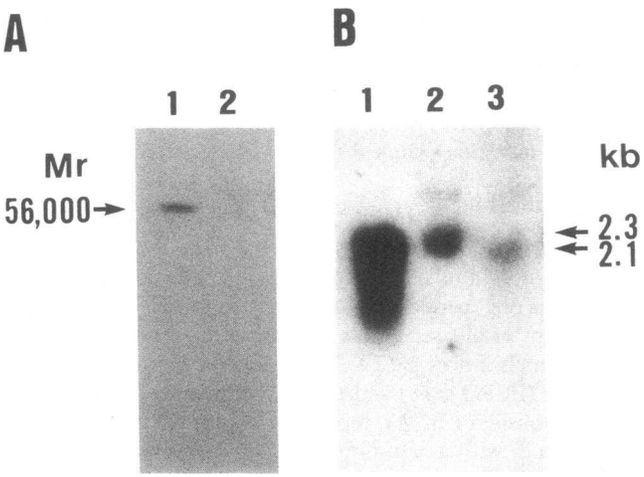

Figure 1. Immune blot analysis (*A*) and Northern blot analysis (*B*) of human prolidase. (*A*) The cell extracts (100 μg of protein) from control lymphoblastoid cells (lane *1*) or patient's lymphoblastoid cells (lane *2*) were subjected directly to gel electrophoresis, as described in Methods. An arrow indicates the 56-kD protein of human prolidase. (*B*) Northern blot analysis of RNA obtained from human placenta and cultured lymphoblastoid cells. RNA was prepared and analyzed as described in Methods. (Lane *1*) 10 μg of poly $(A)^+$ RNA from normal human placenta. (Lane *2*) 10 μg of total RNA from control lymphoblastoid cells. (Lane *3*) 10 μg of total RNA from the patient's lymphoblastoid cells. Arrows indicate size of the mRNA corresponding to the subunit of prolidase.

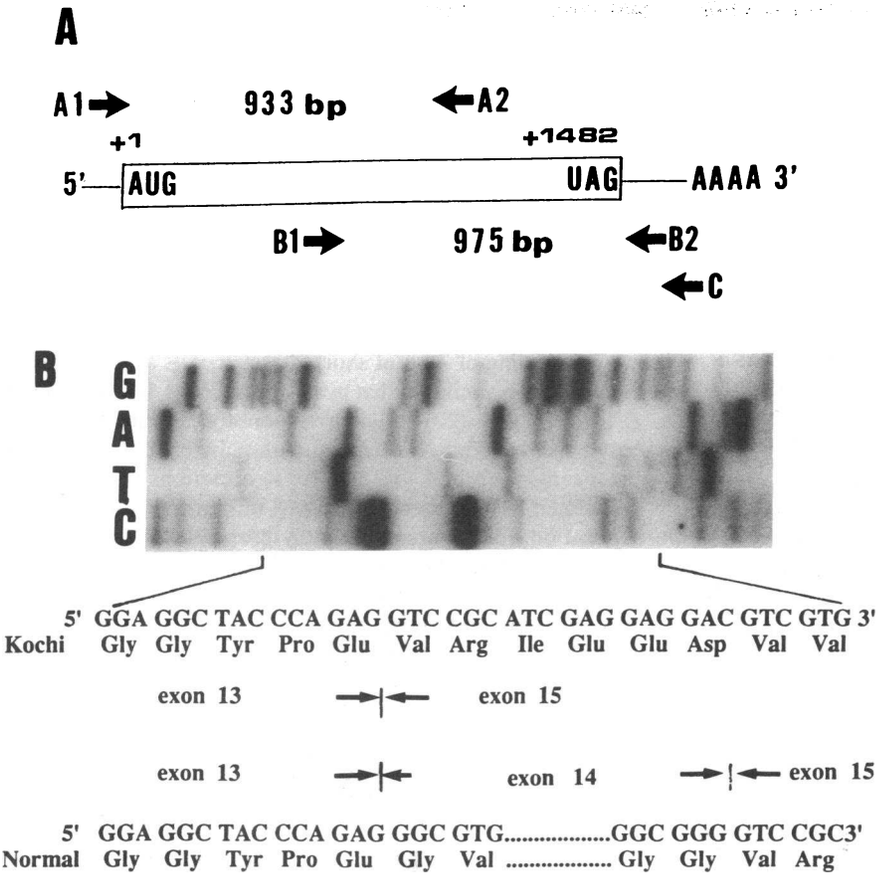

Figure 2. Strategy for PCR amplification (*A*) and the nucleotide sequence of the mutant prolidase cDNA (prolidase Kochi) corresponding to exon 14 (*B*). (*A*) The PCR primers (A1, A2, B1, and B2) were used to amplify prolidase sequences from specific primer C primed DNA:RNA hybrid. The relative priming sites and direction of extension for each primer (*arrows*) are indicated. Sequences of each primers are complementary to those of prolidase and are as follows: A1 (−16 ~ 4); 5'CCGGTGCCGGGCGAACATGG3', A2 (958 ~ 977) 5'CACCAGTCACCTGGCTTCAT3', B1 (664 ~ 683) 5'TTGGAAAGCCTCTTCGAGCA3', B2 (1620 ~ 1639) 5'CCCGGGAAACAGCACTGTTT3', C (1659 ~ 1678) 5'GTGGTGCCTGCAAAAGGGTA3'. Of these, A2, B2, and C represent antisense sequences. Two segments, 933- and 975-bp long, were amplified with primer sets of A and B, respectively. The base number for the A of the ATG start codon and the terminal G of the UAG stop codon are indicated. (*B*) Partial nucleotide sequence of the mutant prolidase cDNA (Kochi). An autoradiograph of the nucleotide sequence of the mutated region is shown. As the normal sequence (3) is indicated in the lower panel, the mutant cDNA (Kochi) skips exon 14, which corresponds to a 192-bp in-frame deletion in the coding region.

lane *1*). Size of the endogenous prolidase was similar to that of human prolidase purified from erythrocytes (Fig. 3 *A*, lane H).

To characterize the proteins expressed in the BMT-10 cells, we attempted to differentiate the human and monkey enzymes, using a series of monoclonal antibodies developed against human enzyme (17). We found that a mouse monoclonal antibody, named mouse IgG-EP38 could immune-precipitate human enzyme but not monkey enzyme. Thus, using the mouse IgG-EP38, we could immunologically differentiate the two enzymes. In addition, the IgG-EP38 could immune-precipitate the mutant protein. We partially purified normal and mutant human prolidases from cell extracts, using immunochemical procedures (17, 21). The partially purified fraction did not contain the monkey enzyme. When the partially purified enzymes from cells transfected with pEPD-W and pEPD-K were analyzed on SDS-PAGE and immune blot, protein bands with M_r 56,000 and 49,000 were visualized, respectively (not shown). The same fraction obtained from cells transfected with pCAGGS did not contain any protein immunologically related to prolidase. The catalytic activities of the partially purified protein are shown in Fig. 3 *B*. The activity of human prolidase associated with cells transfected with pEPD-W was 167 nmol/mg per min and the enzymatic activity associated with cells transfected with pEPD-K was similar to that in case of transfection with the expression vector alone (less than 5 nmol/mg per min). Thus, the abnormal mRNA encoded a protein with a molecular mass of 49 kD and which immunologically cross-reacted with the anti-prolidase antibody. The mutant protein was enzymatically inactive.

To determine whether the skipping of exon 14 in mRNA was due to a mutation at a splicing site or to a gene deletion, including exon 14, we analyzed genomic DNAs from the family members by Southern blot analysis using specific genomic fragments as probes. Data obtained using as a probe an 0.8-kb Pst I fragment of genomic DNA are shown in Fig. 4. A normal 1.5-kb Taq I fragment (Fig. 4, lane *N*) was absent in lanes where the patients' DNA were analyzed. On the other hand, a 1.3-kb fragment was present in cells from the patients (Fig. 4). The parents carried both the 1.5- and the 1.3-kb Taq I fragments. When the nuclear DNA was digested with Pst I and probed with the same fragment a 0.8-kb band was visible in lanes with samples from the control and from the parents, although the intensity of the signal was weak in the latter. The 0.8-kb band was absent in cases of the affected siblings. These results suggested that there was a partial gene deletion around exon 14, and that the affected siblings were homozygotes for the mutant allele. The consanguineous parents were heterozygotes for both normal and mutant alleles.

To determine the actual size of the deletion as well as the precise 5' and 3' borders, we amplified genomic DNAs from the patients and the control (Fig. 5 *A*.) A 3.8-kb fragment was amplified with the primer set D in the control and a 3.0-kb fragment in the patient (Fig. 5 *B*). In another PCR experiment with primer set E, a 1.5-kb fragment was amplified in the control and a 0.7-kb fragment in the patients (not shown). The deletion was ~ 0.8 kb long.

We determined the nucleotide sequence of most of intron 13, exon 14, and intron 14 of the normal allele and of the boundary of the deletion junction of the mutant allele. We found that the span between the 5' and 3' breakpoints was 774

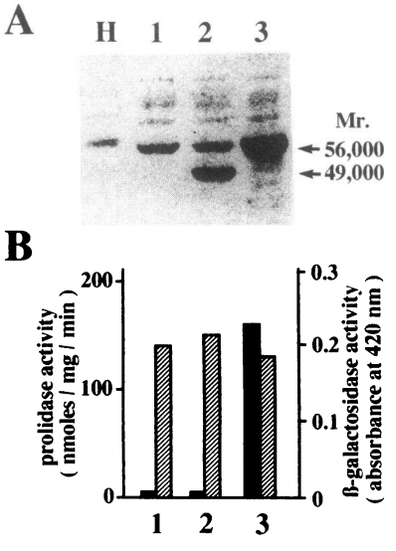

Figure 3. Immune blot analysis (*A*) and specific enzyme activities (*B*) of human prolidase in transfected BMT-10 cells. Plasmid pCH110 was cotransfected as an internal standard to normalize for any variability in the transfection efficiency. BMT-10 cells were transfected with 10 μg of prolidase cDNA and/or 5 μg pCH110 cDNA using 30 μg of lipofection, then harvested on the third day. (*A*) Proteins (100 μg) from BMT-10 cells transfected with pCH110 + pCAGGS (lane *1*), pCH110 + pEPD-K (lane *2*), and pCH110 + pEPD-W (lane *3*) were subjected to gel electrophoresis. Purified prolidase protein from human erythrocytes were analyzed for comparison (lane *H*). Arrows indicate the 56-kD protein of human prolidase and/or endogenous prolidase from BMT-10 cells, and the 49-kD of mutant prolidase. (*B*) Specific prolidase activities expressed in the transfected BMT-10 cells (1, pCH110 + pCAGGS; 2, pCH110 + pEPD-K; 3, pCH110 + pEPD-W). Immune precipitation by monoclonal antibody was performed as described in Methods. The enzyme activities that bound to mouse IgG EP38 were measured and are expressed as nanomoles proline per milligram protein per minute and are indicated by black bars. The activities of β-galactosidase in the cell extracts (100 μg of protein) transfected with pCH110 were measured to monitor the efficiency of transfection and are defined as the amount of product formed per hour, as determined spectrophotometrically (*hatched bars*).

bp long and that 7 bp direct repeats (CCACCCT) were present at the 5′ and 3′ breakpoints (Fig. 5 *C*).

Discussion

Molecular analyses revealed that two Japanese siblings, with or without skin lesions, have the same gene deletion. An immunological protein of 49 kD which cross-reacted with the anti-human prolidase antibodies was present in cells transfected with the mutant expression clone (pEPD-K). The estimated size of the mutant subunit polypeptide from the calibrated SDS-PAGE is in a good agreement with the calculated value of 47,200, based on the predicted amino acid sequence. Partial purification and specific enzyme assay of the human prolidase provided evidence that the abnormal prolidase is enzymatically inactive. Although the active site of the enzyme is unknown, the absence of 64 amino acid residues may result in a local change affecting the active site and/or the region which participated in dimer formation.

Our initial investigations on cultured fibroblasts (7) and cultured lymphoblastoid cells (Fig. 1) indicated that the immunological protein related to prolidase was absent in cells from the siblings. These results suggested that the shortened, inactive, and less stable protein is synthesized in the patients' cells. A markedly reduced activity of the mutant enzyme and the lower level of the enzyme protein seem to be linked to the loss of prolidase activity, in this family. The parents carried both the mutant-type and the wild-type allele for prolidase gene, thus it seemed likely that both the normal and the abnormal subunits of prolidase were synthesized. If the mutant subunit remained long enough to compose a heterodimer with the normal subunit and the heterodimer was inactive, the apparent activity of prolidase in the parents' cells would be lower than the value expected as a carrier. However, the enzyme activity in the parents' cells was ~ 50% of the control value, hence, the abnormal subunit protein might not be functional in the parents' cells and the heterodimer of the normal and abnormal subunits might not be formed.

The finding of 7 bp of short, direct repeats at the deletion breakpoints is similar to that noted in studies of deletions of the *E. coli* lac I gene (22) and the retinoblastoma gene (23). In the latter, the deletions had termini with pairs of short, direct repeats ranging from 4 to 7 bp. These mutations (22, 23) were speculated to be caused by "slipped mispairing" during DNA replication. In this model, initially proposed by Streisinger et al. (24) to explain the generation of frame shift mutations, the repeated sequences mispair during DNA replication, leading to the formation and excision of a single-stranded loop between the repeats. One repeat present in the loop is then deleted along with the segment between the repeats, and one repeat remains

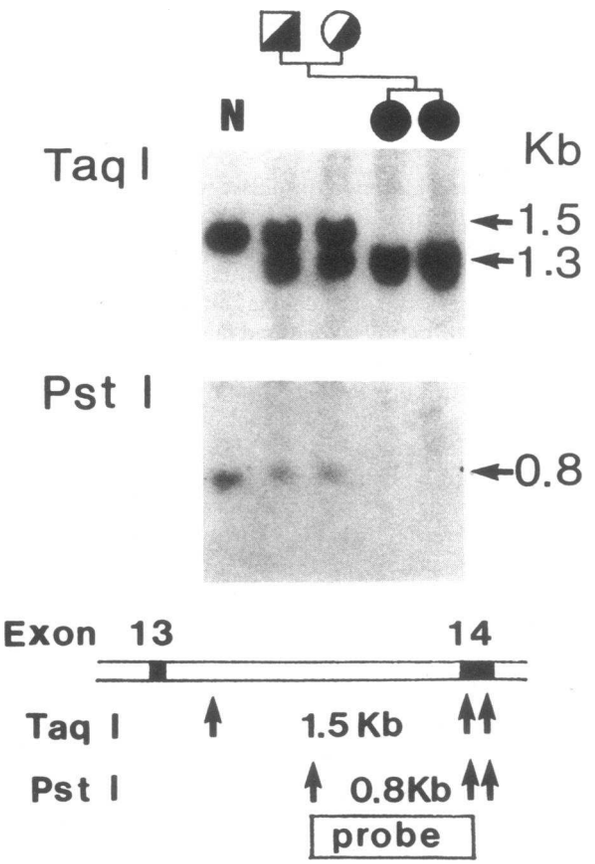

Figure 4. Southern blot analysis of genomic DNAs from siblings with prolidase deficiency and the control, using a 0.8-kb Pst I genomic fragment containing part of intron 13 and exon 14. The partial structure of the gene and the probe used are shown in the lower panel. Taq I and Pst I digested DNA (10 μg) from the control (lane *N*) and the pedigree were hybridized using the same probe.

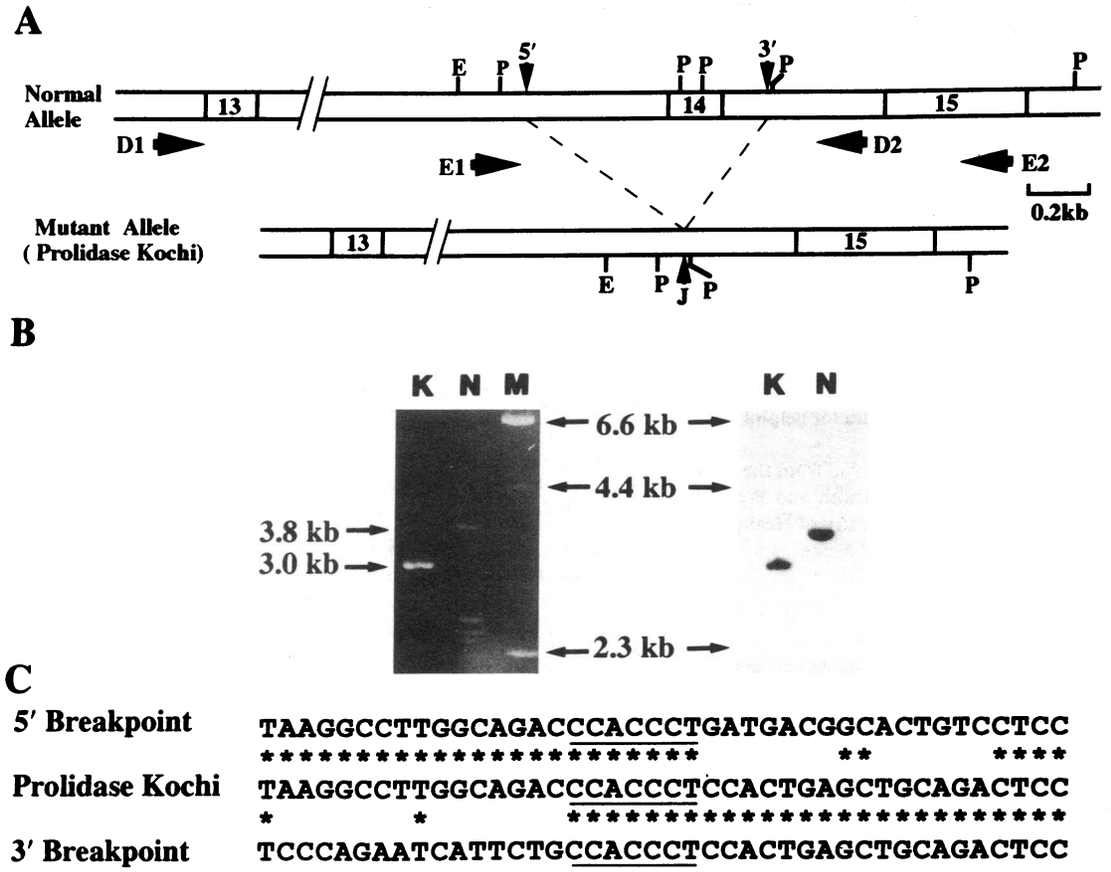

Figure 5. (*A*) Restriction maps of the mutant prolidase junction fragments and the fragment containing the 5′ and 3′ deletion breakpoint, and primers (D1, D2, E1, and E2) for PCR of genomic DNA. The relative priming sites and direction of extension for each primer (*arrows*) are indicated. Numbers in the box indicate exon numbers. Abbreviations: *E*, Eco RI; *P*, Pst I; *5′*, 5′ breakpoint; *3′*, 3′ breakpoint; *J*, deletion junction. (*B*) Photograph of gel electrophoresis of the PCR products. Ethidium bromide stain (*left*) and Southern blot analysis using as a probe, prolidase cDNA (*right*). PCR of genomic DNA was performed, using primers as follows. The 5′ primer (*D1*) was a 25-mer (TTGGATCCCCAGTACC-CACTTGCCC3′) that contained 17 bases of the intron 12 sequence. The 3′ primer (D2) was a 20 mer (5′TCACCTGCTCACCCTGTCTT3′) corresponding to the sequence in intron 14. With the primer set, a 3.8- or 3.0-kb fragment was amplified in control (lane *N*) or patient's DNA (lane *K*), respectively. Molecular size marker is indicated in lane *M*. The fragment was specifically hybridized to the cDNA probe. Another PCR was performed using primer set E. The 5′ primer (*E1*) was 20 mer (5′CACGGAAAACCTGGGCCTCG3′) corresponding to the sequence in intron 13, and the 3′-primer (*E2*) was 20 mer (5′CCCGGGAAACAGCACTGTTT3′), corresponding to the sequence in exon 15. (*C*) Sequence of the deletion junction compared with the 5′ and 3′ breakpoints. A 7-bp direct repeat that is present at both breakpoints and at the deletion junction is underlined. Asterisks represent identical nucleotides. (Kochi is the name of a city.)

in the mutant DNA. The "slipped mispairing" mechanism may explain the deletion in this Japanese family with prolidase deficiency.

Gene analysis indicated that the mutant gene with the deletion was inherited from the consanguineous parents. Thus, prolidase deficiency is a genetic disorder which obeys the low of Mendel. In the present study, we analyzed findings in siblings with the polypeptide negative phenotype of prolidase deficiency. These siblings had a similar degree of defects in imidodipeptide metabolism, as deduced from urine analyses of the imidodipeptides. However, the clinical phenotypes in these siblings markedly differed.

Our initial objective in examining this unique family with prolidase deficiency, with and without of the clinical symptoms was to learn more about the molecular mechanisms underlying defects in this enzyme. Our data clearly show that the siblings with prolidase deficiency carry the same mutation in the PEPD gene. In addition, the parents are heterozygotes for the mutant allele. Thus, even detailed molecular analysis of the family provided no simple explanation for the difference in clinical symptoms between the siblings. To elucidate the different severities of clinical symptoms in patients with the same defect, factor(s) not related to the PEPD gene product and which influence the development of skin lesions, especially age at onset, need to be given attention. Other investigators reported familial cases of prolidase deficiency in which a female proband with clinical symptoms and her brother without clinical symptoms had a similar degree of enzyme defects and imidodipeptiduria (25). Imidopeptide hydrolyzing activity other than prolidase in human cells (21, 26) was a putative candidate for such a factor, however, we ruled out this possibility (7).

Prolidase deficiency is a chronic disorder and various symptoms appear with time. Histological examination of the affected skin and a severe ulcer revealed amyloidlike substance in the epithelial cells and in the lumen of the blood vessels, unidentified substances were present (27). Thus, mechanisms involved in the development of skin ulcer, the most severe instance of skin lesions in prolidase deficiency, might be a com-

plicated process involving epithelial, endothelial, and other cells. Environmental factors could also modify the development of skin lesions.

Further identification and characterization of the mutations of PEPD gene will not only provide important insight into the physiological role of prolidase and its structure-function relationship but also enhance our understanding of mechanisms underlying the development of clinical symptoms.

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