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A New Allele, *DNASE1*6,* of Human Deoxyribonuclease I Polymorphism Encodes an Arg to Cys Substitution Responsible for Its Instability

Toshihiro Yasuda,* Haruo Takeshita,* Reiko Iida,† Shinya Kogure,* and Koichiro Kishi*,[1]
**Department of Legal Medicine, Gunma University School of Medicine, Maebashi, 371-8511, Japan; and*
†*Department of Legal Medicine, Fukui Medical University, Matsuoka-cho, 910-1159, Japan*

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A new allele, *DNASE1*6,* of human deoxyribonuclease I (DNase I) has been discovered by isoelectric focusing: its gene product has the most cathodic p*I* of the six electrophoretic variants. Results of DNA sequencing, mismatched PCR-restriction fragment length polymorphism, and transient transfection of the variant construct showed that the mutant was caused by a C–T transition at nucleotide position 1826, resulting in an Arg to Cys substitution at amino acid position 185 of the mature enzyme. The variant isoenzyme, expressed in COS-7 cells, was more labile than the other types. Instability and an increase in the p*I* value of the variant suggest that a structural alteration, perhaps due to aberrant formation of a disulfide bond, could occur in the enzyme.

Deoxyribonuclease I (DNase I; EC 3.1.21.1) or DNase I–like enzymes have been postulated to be responsible for internucleosomal DNA degradation during apoptosis (1, 2). The presence of DNase I activity in mammalian tissues other than the digestive organs suggests further *in vivo* functions (3–6). We first reported that human DNase I exhibits genetic polymorphism, indicated by differences between the pI values of type-specific isoenzymes revealed by isoelectric focusing (IEF) (7). At a single autosomal locus assigned to band 16p13.3 (8), five codominant alleles—*DNASE1*1, *2, *3, *4,* and *5*—have been identified so far (7, 9), and the molecular basis has been elucidated (9–12). Not all of the amino acid residues underlying the genetic polymorphism may be part of any putative functional sites of human DNase I, and no significant type-related variations in the specific activity of DNase I have been observed (3, 9, 11, 12). These findings allow us to postulate that these amino acid substitutions at polymorphic sites may not affect the specific functions of the enzyme. It was reported that a hereditary abnormal species of human plasminogen retained its antigenic activity but no enzymatic activity, due to maybe structural alterations around the active site of the enzyme (13). However, no variant of DNase I that exhibits such a discrepancy between its antigenic and enzymatic activities has been discovered so far. Furthermore, inherited deficiency of the enzyme is not yet known to be associated with any disease.

In this paper, we describe the identification of a new allele, *DNASE1*6,* based on a C–T transition in exon 7 of the gene and transient expression of the mutant construct in COS-7 cells. This is the first report to elucidate a considerably labile species of human DNase I.

MATERIALS AND METHODS

DNase I phenotyping. Urine samples were collected from more than 1500 healthy Japanese subjects, and DNase I was phenotyped by the method of Yasuda *et al.* (14). After electrophoresis, the DNase I isoenzyme patterns were observed by immunoblotting with an anti-human urinary DNase I antibody. Serum DNase I was detected by the dried agarose film overlay (DAFO) activity staining method (7, 14) and made visible with a LAS-1000 system (Fuji Film, Tokyo, Japan). The DNase I activity in the samples was determined by the single radial enzyme diffusion (SRED) method (3).

PCR-amplification and the subsequent analysis. Genomic DNA was isolated from peripheral blood leukocytes using a QIAamp blood kit (Qiagen, Chatsworth, CA). Two partially overlapping fragments, PCR-5 and -3, which included entire exons 2–5 and 6–9, respectively, were amplified and subjected to direct DNA sequencing using sequencing primers suitable for each exon according to the method of Yasuda *et al.* (10, 12). Sequencing was done with a DNA sequencer (Model 310, Applied Biosystems, Urayasu, Japan). The PCR-3 fragment, containing exon 7, was subcloned directly into a TA cloning pCR II vector (Invitrogen, San Diego, CA), and twelve independent subclones were isolated and sequenced.

[1] To whom correspondence should be addressed. Fax: +81-27-220-8035. E-mail: kkoichi@news.sb.gunma-u.ac.jp.

Abbreviations used: DAFO, dried agarose film overlay; DNase, deoxyribonuclease; IEF, isoelectric focusing; RFLP, restriction fragment length polymorphism; SRED, single radial enzyme diffusion.

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Confirmation of a mutation site by a mismatched PCR-restriction fragment length polymorphism (RFLP) analysis. Fragments including the mutation site were amplified using the following primers: MIS6-1 (5′-ATGTGAGACCCTCCCAGTGTCATAGAT-3′, sense primer), which corresponds to nucleotide positions 1797–1824 in the published human DNase I gene sequence (10) except that AG was substituted for CC at positions 1821-2, and D-4 (5′-GAGTCACTTCTGCCCAAGG-3′, antisense primer), which corresponds to positions 1952–71. After PCR-amplification, 10 μl of PCR products was digested with 20 units of *Bgl*II at 37°C for 2 h and then subjected to 10% polyacrylamide gel electrophoresis followed by silver-staining.

Transient expression of the type 6 construct in COS-7 cells. A DNA fragment containing the entire coding sequence for human type 2 DNase I cDNA was prepared from total RNA derived from a pancreas by reverse-transcriptase PCR amplification by using a set of two primers, 5′-AAAGGATCCTCTCAGGATGAGGGGCATGAAG-3′ and 5′-AAAGCGGCCGCCTCACTTCAGCATCACCTCCAC-3′ (15). The pancreas came from a 48-year-old man (type 2) 12 h after death due to loss of blood. The fragment was ligated into a pcDNA3.1(+) vector (Invitrogen) to construct the expression vector DN1-ty2. For site-directed mutagenesis of the mutation site in the type 6 isoenzyme, a mutant cDNA construct (DN1-ty6) was produced by the overlap extension method (16) using the DN1-ty2 as a template. All constructs were sequence-confirmed and were purified by using the Plasmid Midi kit (Qiagen) for transfection. COS-7 cells were maintained in Dulbecco's modification of Eagle's medium containing 1 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) fetal calf serum (Life Technologie Inc., Gaithersburg, MD) at 37°C under 5% CO_2 in air. The cells were transiently transfected using Lipofectamin Plus reagent (Life Technologie Inc.) according to the manufacturer's instructions. Two days after transfection, the medium was recovered for subsequent analysis.

RESULTS

Elucidation of a new electrophoretic variant "type 6." A rare electrophoretic variant of DNase I was observed by IEF-analysis in one urine sample; the isoenzyme has the highest pI value of the six DNase I isoenzymes identified so far (Fig. 1A). It was clarified that the allele was transmitted from the donor's (propositus') mother. We designated this new allele *DNASE1*6.* The DNase I phenotypes of the propositus, his brother, and his mother were determined to be 2-6, 1-2, and 1-6, respectively. The band of the type 6 isoenzyme detected by immunostaining was fainter than those of the other types. The DNase I activities in the subjects' sera were 1.25 ± 0.21 and 1.59 ± 0.22 units/liter, being approximately half those of the other phenotypes (3). Notably, although the conventional DAFO activity staining conditions allowed the other isoenzymes to be distinctly observed, the band corresponding to type 6 was barely detected in these sera (Fig. 1B).

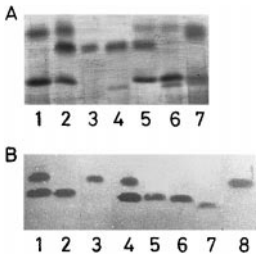

FIG. 1. IEF (pH 3.5–5) patterns of DNase I phenotypes in (A) urine and (B) serum samples made visible by immunoblotting and activity staining, respectively. (A) Urine samples collected from the propositus and his family were subjected to IEF, followed by immunoblotting with anti–human DNase I. Lane 1, type 2; 2, type 1-2; 3, type 1; 4, type 1-6 (propositus' mother); 5, type 1-2 (propositus' brother); 6, type 2-6 (propositus); 7, type 2-5. (B) DNase I isoenzyme patterns of serum samples and the culture medium of cells transfected with type 2 (lane 6) and type 6 (lane 7) constructs were made visible by DAFO activity staining, following IEF. Types 2 and 6 constructs were transiently expressed in COS-7 cells, and each of their medium was recovered. Lane 1, type 1-2; 2, type 2; 3, type 1-6 (propositus' mother); 4, type 1-2 (propositus' brother); 5, type 2-6 (propositus); 8, type 1. Electrophoretic and staining conditions are described under Materials and Methods. Anode is at the top.

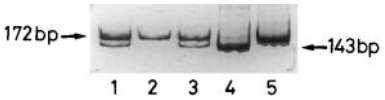

FIG. 2. Detection of the C1826T substitution in exon 7 underlying the *DNASE1*6* allele by mismatched PCR-RFLP analysis. Mismatched PCR amplification of genomic DNA from the propositus (lane 1), his brother (lane 2) and his mother (lane 3) was done by using a set of primers, MIS6-1 and D-4. After digestion of the amplified products with *Bgl*II, the digests were electrophoresed on 10% polyacrylamide gel and made visible by silver-staining. Plasmids inserted with *DNASE1*6* (lane 4) and *DNASE1*2* (lane 5) were used as controls.

*Identification of the nucleotide substitution underlying DNASE1*6.* Direct DNA sequencing of the PCR-amplified products covering the entire open reading frame of DNase I from the propositus and his mother revealed only one heterozygous nucleotide substitution: a C to T transition at position 1826 in exon 7. To confirm this transition, the amplified PCR-3 fragment containing exon 7 from the propositus (type 2-6) was subcloned. The 12 independent subclones were separately subjected to DNA sequencing: six had T at 1826, and the rest had C, corresponding to the *DNASE1*2* allele, consistent with the direct sequencing analysis. The same transition at position 1826 was confirmed for the mother (type 1-6).

This C1826T substitution neither suppressed nor created any known site for restriction enzyme recognition, and we therefore designed a mismatched PCR primer (MIS6-1) to create a new *Bgl*II recognition site (AGATCT). A 172 bp DNA fragment containing the mutation site was amplified using primers MIS6-1 and D-4. If the C1826T mutation were present, the mismatched PCR-amplified product should be degraded with *Bgl*II to fragments of 29 and 143 bp. If the other alleles were present, no new *Bgl*II site would be yielded. The mismatched PCR-RFLP analysis was applied to each amplified product from the propositus and his family (Fig. 2). Both amplified products derived from the propositus (type 2-6) and his mother (type 1-6)

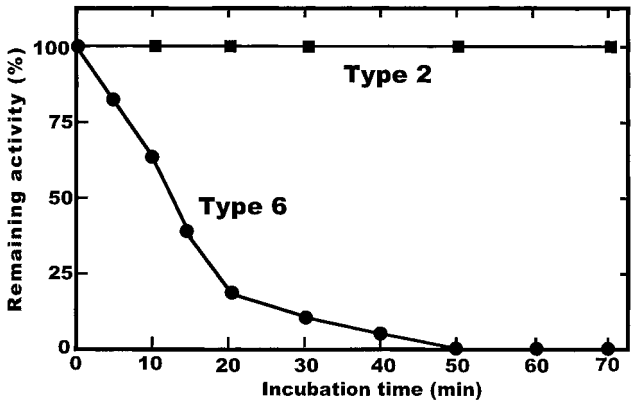

FIG. 3. Heat stability of the type 6 isoenzyme. Each medium of cells transfected with the type 2 and type 6 constructs was incubated at 50°C, and their DNase I activities were determined by the SRED method. Even at 37°C, the time required for the activity of the expressed type 6 to be reduced to half its original value was approximately 6 h.

had two bands (172 and 143 bp) and were clarified to be heterozygous for the C1826T mutation, consistent with results of the DNase I phenotyping by IEF. Therefore, this mutation resulted in the substitution of Arg (codon CGC) by Cys (codon TGC) at amino acid position 185 of the mature enzyme.

Expression of type 6 in mammalian cells. The entire coding regions of types 2 and 6 DNase I cDNA were cloned into mammalian expression vectors (DN1-ty2 and -ty6, respectively) and transiently expressed in COS-7 cells. The DNase I activities (± SEM, $n = 6$) secreted in the medium were 3.12 ± 0.980 and 0.748 ± 0.139 units/ml, respectively, and were completely abolished by the anti-human DNase I antibody. When the expressed DNase I isoenzyme patterns were stained by the DAFO activity staining in IEF, each isoenzyme derived from the DN1-ty2 and -ty6 constructs migrated to the positions on the gels corresponding to the types 2 and 6 isoenzymes in the urine samples (Fig. 1B). Expression of each construct in human hepatoma Hep G2 cells in place of COS-7 gave similar results (data not shown). These findings lead us to conclude that a C to T substitution at position 1826 underlies *DNASE1*6* allele.

Next, we examined heat stability by preincubating the expressed isoenzymes at 50°C for various periods (Fig. 3). In contrast to the type 2 isoenzyme, the activity of type 6 isoenzyme was reduced to about 50% of the original activity after about 10 min incubation. The other isoenzymes exhibited heat stability similar to type 2. These results indicate that the type 6 isoenzyme is more labile than the other types, which is consistent with the observation that the activity stained band corresponding to type 6 in the propositus and his mother's sera could not be made visible on the activity stained gels (Fig. 1B).

DISCUSSION

Sequence analysis of exon 7 from DNase I types 1-6 and 2-6 reveals that the substitution of C by T at nucleotide position 1826 is responsible for the *DNASE1*6* allele and leads to the replacement of Arg by Cys at amino acid position 185 (R185C). We have already identified the nucleotide substitution sites at positions 91 (12), 1227 (9), 1592 (11), and 2317 (10) underlying the other alleles; all residues at each of these sites in the *DNASE1*6* allele were identical to those in the *DNASE1*2* allele. The amino acid sequence of the variant was also different from that of type 1 by only two residues, at positions 185 (R185C) and 222 (Q222R). The type 6 isoenzyme had the highest pI value. However, in comparison with type 2, the substitution of Arg by Cys at position 185 in type 6 led to a change in the charge state of the enzyme protein, theoretically resulting in a decrease in its pI value. In fact, the pI values of types 1, 2, and 6 enzyme proteins were predicted by using GENETYX-MAX (Software Development Co.) to be 4.37, 4.43, and 4.37, respectively. Recently, we reported that, irrespective of replacement of Val at position 92 in the type 2 isoenzyme by Met, a concomitant change in conformation results in a slight increase in the pI of type 5 (9). When the secondary structure of the types 2 and 6 isoenzymes was predicted by the method of Chou and Fasman (17), little perturbation of this substitution on the protein structure could be observed. Four Cys residues at positions 101, 104, 173, and 209 are conserved in mammalian DNase I enzymes. Two disulfide bonds are formed between Cys^{101}–Cys^{104} and Cys^{173}–Cys^{209}, the latter of which has been shown by the X-ray structure analysis to contribute to the stability of the enzyme by (18, 19). As shown in Fig. 3, the amino acid substitution (R185C) in the type 6 isoenzyme made the enzyme much less stable than the other types. The C1826T substitution in the *DNASE1*6* allele results in an additional Cys residue between the two essential Cys residues at 173 and 209. It is reasonable to assume that involvement of the "new" Cys residue at position 185 in the formation of a disulfide bond with Cys^{173} or Cys^{209} might not permit the enzyme to obtain intrinsic structural stability and rigidity because of a loss of the essential disulfide bond Cys^{173}–Cys^{207} responsible for the stability of the enzyme. These structural alterations are evident from the unexpected shift of the electrophoretic mobility of the type 6 isoenzyme and the instability of the enzyme. Further investigation may be needed to shed light on the structural alterations.

Notably, as all the other types so far identified exhibit similar stability and activity levels in human sera, the type 6 isoenzyme is the first type to be shown to be labile. We have previously proposed that the *DNASE1*2* and **3* alleles must have been produced by

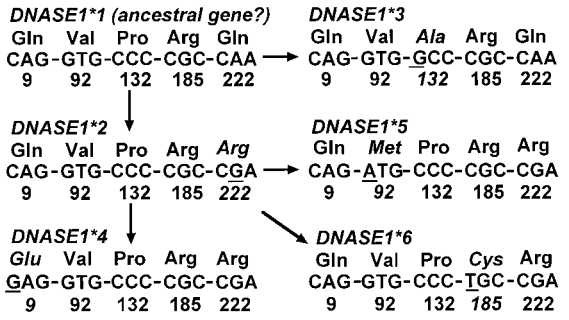

FIG. 4. Probable molecular basis for the genesis of polymorphism in human DNase I. The amino acid residues responsible for the genetic polymorphism of DNase I are numbered starting with the amino-terminal residue. Considering that the *DNASE1*1* allele occurs at the highest frequency in the Japanese population (20), and that Gln at position 222 is conserved in other mammals, it is plausible that the *DNASE1*1* allele is the ancestral monomorphic gene. The sites of the nucleotide substitution responsible for generation of each allele are underlined. The amino acid residues resulted from these nucleotide substitutions are numbered starting with the N-terminal residue of the mature enzyme.

point mutation in exons 8 and 6, respectively, of the ancestral *DNASE1*1* allele, and that point mutations in exons 2 and 5 of the *DNASE1*2* allele must give rise to the *DNASE1*4* and **5* alleles, respectively (12). According to this hypothesis, the *DNASE1*6* allele may have been derived from the *DNASE1*2* allele by point mutation in exon 7 (Fig. 4). As inherited deficiency of the enzyme is not yet known, it would be interesting to examine whether a homozygote of *DNASE1*6* allele might be associated with any disease.

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