Volume 301, number 1, 34–36 FEBS 10910 April 1992

A dysfunctional C1 inhibitor protein with a new reactive center mutation (Arg-444→Leu)

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Received 17 February 1992

A P1 mutation (Arg-444→Leu) was identified in a dysfunctional C1 inhibitor from a patient with type 2 hereditary angioneurotic edema. The mutation was defined at the level of the protein (by sequence analysis of the *Pseudomonas aeruginosa* elastase-derived reactive center peptide), and the mRNA (CGC→CTC) (by sequence analysis of PCR-amplified DNA).

C1 inhibitor; Hereditary angioneurotic edema; Serpin; Point mutation; Plasma proteinase inhibitor

1. INTRODUCTION

C1 inhibitor (C1 INH) is a member of the *ser*ine *p*roteinase *in*hibitor (serpin) superfamily. The reactive center region of serpins mimics the natural substrate of target proteases. The residue amino-terminal to the peptide bond recognized by target proteases is termed P1, while P1′ is the residue carboxyl-terminal to this bond. Adjacent residues in each direction are numbered consecutively. The 'attempt' by the protease to hydrolyze the P1–P1′ bond is subverted by the formation of a tight protease–protease inhibitor complex. A primary determinant of inhibitor specificity is the P1 residue, which, in the case of C1 INH, is arginine.

Dysfunction, or deficiency, of C1 INH results in hereditary angioneurotic edema (HANE). The majority of mutations resulting in dysfunction of C1 INH (type 2 HANE) alter the codon for the reactive center arginine (P1) [1–4]. Single base changes in this codon (CGC) could result in six possible amino acid substitutions: His, Cys, Ser, Leu, Gly or Pro. Nearly all P1 mutations thus far observed replace the Arg with His or Cys. One example of a P1 Arg→Ser has been described [4]. The preponderance of His and Cys substitutions is likely related to the increased propensity toward mutation of the cytosine in the CpG dinucleotide [5]. In this communication we describe the analysis of dysfunctional C1 INH (Gu), in which the P1 Arg is replaced with a Leu residue.

Abbreviations: C1 INH, C1 inhibitor; HANE, hereditary angioneurotic edema; PCR, polymerase chain reaction; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Patient*

The patient is a member of a kindred in which no other family members have symptoms suggestive of HANE. She, therefore, may have a spontaneous new mutation. Unfortunately, family members were not available for testing, so this could not be proven formally. No disease-associated restriction fragment length polymorphism was observed in this patient [6].

2.2. *Polymerase chain reaction (PCR) amplification*

Total cellular RNA was isolated from monocyte monolayers [7] as previously described [8], and was quantitated by absorbance at 260 nm. cDNA was synthesized from the patient's and normal RNA (1 μg) with AMV reverse transcriptase (Boehringer-Mannheim Inc., Indianapolis, IN). The cDNA was amplified by PCR using *Taq*I DNA polymerase (Perkin-Elmer Corp., Norwalk, CT; 30 cycles of denaturation, 94°C, 1 min; annealing/extension 72°C, 5 min [9]). Three sets of sense/antisense 30-base oligonucleotides were synthesized (Applied Biosystems model 380B DNA synthesizer, Applied Biosystems, Foster City, CA), and were designed to cover the entire C1 INH cDNA coding sequence. Amplified cDNA was purified by agarose gel electrophoresis followed by electroelution (International Biotechnologies Inc., New Haven, CT).

2.3. *DNA sequencing*

Amplified DNA was subcloned into pUC19 (Boehringer-Mannheim Inc.) and double-stranded DNA sequencing was performed by the dideoxynucleotide chain-termination method (Sequenase Kit; United States Biochemical Corp., Cleveland, OH) [10].

2.4. *C1 INH isolation and analysis*

C1 INH was isolated from Gu plasma by lectin affinity chromatography [11]. The isolated protein was digested with *Pseudomonas aeruginosa* elastase (a gift of Dr. Morihari, Kyoto Research Laboratories, Tokyo, Japan) as described by Pemberton et al. [12]. High perform-

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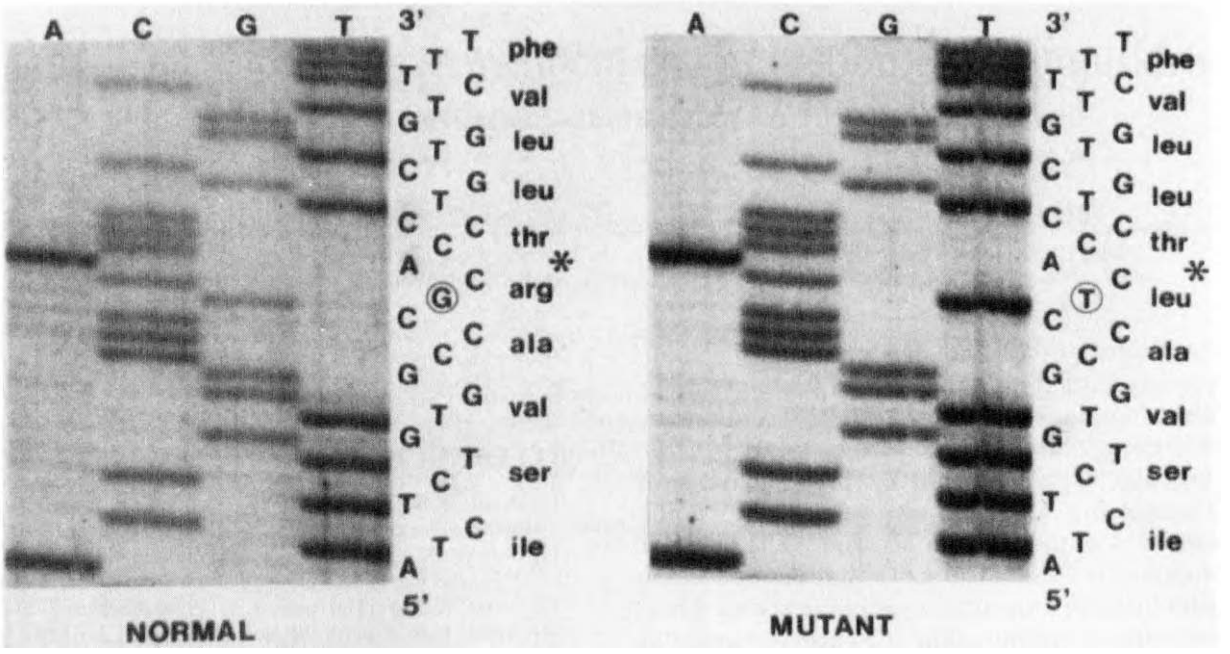

Fig. 1. Nucleotide sequence analysis of the PCR-amplified Gu DNA from the normal and mutant alleles. The sequences shown each corresponds to the coding strand. The protein sequence, therefore, from bottom to top corresponds to amino- to carboxy-terminal. The altered nucleotides are circled, and the resulting amino acids indicated by asterisks.

ance liquid chromatography (HPLC) separation of the *Pseudomonas* elastase-derived peptides was performed as described by Aulak et al. [1] using a reverse phase column (Waters Deltapak C18, 300 Å, 3.9 mm × 15 cm). After sample injection, the column was washed with 0.1% trifluoroacetic acid (TFA)/5% acetonitrile for 10 min, following which a linear gradient to 0.1% TFA/80% acetonitrile was developed; flow rate was 1 ml/min. Amino terminal sequence analyses of the isolated peptides were performed using an Applied Biosystems 477A protein sequencer with 610 data analysis software (Foster City, CA).

3. RESULTS

3.1. *DNA sequence analysis*

Sequence analysis of PCR-amplified DNA revealed the presence of clones that contained a single base change at nucleotide 1432, as compared with the normal sequence (Fig. 1). This G→T mutation converted the codon for the reactive center Arg-444 to a Leu codon. There were no other changes in the coding sequence of the mutant allele. Clones were also identified in which the sequences were completely normal, which is consistent with the heterozygous nature of HANE. The mutation was confirmed with two separate preparations of PCR-amplified DNA in order to diminish the possibility that the mutation was a PCR-induced artifact.

3.2. *Peptide sequence analysis*

SDS-PAGE of isolated C1 INH Gu revealed a single polypeptide with an apparent M_r of 105,000 (not shown), which was indistinguishable from the normal protein. HPLC separation of the *Pseudomonas* elastase-generated products revealed three peptides peaks that eluted between 33 and 35 min into the gradient. Normal C1 INH protein digested with *Pseudomonas* elastase yielded a single peptide in this region, which resulted from cleavage of the peptide bond carboxy-terminal to Ser-441 (between P3 and P4) [1,12]. In addition, many C1 INH preparations contained the carboxy-terminal fragment resulting from cleavage between P1 and P1′. The first two peptides to elute (at 33.30 and 33.60 min, Fig. 2) were present in relatively low yield. The amino-terminal sequence of the first of these was identical to the sequence beginning with P1′, while the second had a sequence matching that of the peptide derived from *Pseudomonas* elastase cleavage of the normal protein between P3 and P4. The third peptide revealed an amino-terminal Leu followed by a sequence that matched the sequence beginning with P1′. This thus confirms the replacement of Arg-444 with a Leu residue in C1 INH Gu. In addition, it appears that this substitution results in the creation of a new site that is susceptible to proteolysis by *Pseudomonas* elastase. This new site, Ala–Leu, is compatible with the known substrate specificity of *Pseudomonas* elastase.

4. DISCUSSION

Seventy to seventy-five percent of dysfunctional C1 INH proteins analyzed thus far have resulted from replacement of the reactive center Arg residue [1–4,13]. Including the protein described here, of 32 mutant dysfunctional proteins [1–4,13–17; Aulak, unpublished data) 23 had substitutions at the reactive center. Only

	P7				*		P1	P1'							P12
Normal	Ser	Ala	Ile	Ser	Val	Ala	Arg	Thr	Leu	Leu	Val	Phe	Glu	Val	Gln	Gln	Pro	Phe
33.30 min								Thr	Leu	Leu	Val	Phe	Glu	Val	Gln	Gln	Pro	
33.60 min						Val	Ala	Arg	Thr	Leu	Leu	Val	Phe	Glu	Val			
34.05 min								Leu	Thr	Leu	Leu	Val	Phe	Glu	Val	Gln	Gln	

Fig. 2. The amino acid sequence of normal C1 INH in the region extending from P7 to P12', together with the sequences of the three peptides derived from C1 INH Gu. The asterisk indicates the previously determined site of cleavage by *Pseudomonas* elastase.

two were residues other than His or Cys. Most nonreactive center mutant proteins resulted from mutations within the hinge region (residues P9–P14), amino-terminal to the reactive center [13–15,17].

The probable explanation for the preponderance of reactive center Arg→His and Cys substitutions is the hypermutability of the CpG dinucleotide. The C1 INH Arg-444 codon is CGC. The CpG dinucleotide is the primary methylated sequence in vertebrates, and it mutates frequently by deamination of 5-methylcytosine to thymine [18]. This mechanism accounts for as much as 35% of coding region point mutations causing human disease [18–20]. At Arg-444, this mutation in the coding strand (CGC→TGC) results in replacement with a Cys residue. The same mutation in the anticodin (GCG→GTG) results in alteration of the codon to one encoding a His (CAC). Of the other four possible replacements resulting from point mutation at the reactive center, Ser [4] and Leu (this report) each have been observed once, while neither Gly nor Pro have been seen. Although the data remain limited, there are some indications that the number of mutations (including silent point mutations) within exon 8 (which encodes the reactive center and hinge region) may be greater than the number expected if mutations were distributed randomly through the gene [13–17,21,22]. There may therefore be as yet undefined factors (in addition to the CpG dinucleotide) that predispose this region to mutation.

None of the reactive center mutants have significant activity against the normal target proteases. Arg-444→His mutants produced by site-directed mutation revealed the acquisition of inhibitory activity against chymotrypsin [23]. Subsequently, the naturally occurring P1 His mutant was shown to form a complex with, and inhibit chymotrypsin [24]. The normal C1 INH protein also inhibited chymotrypsin, although less efficiently than the His mutant. The normal protein, however, used the P2 Ala as the reactive center rather than the P1 Arg, while the mutant used the P1 His. Because chymotrypsin activity is favored by hydrophobic residues in the P1 position in substrates, it is possible that C1 INH Gu may inhibit chymotrypsin or other chymotrypsin-like proteases. Studies that will analyze the interaction of this mutant with chymotrypsin and cathepsin G are in progress.

Acknowledgements: This work was supported by US Public Health Service Grant HD22082.

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