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Antithrombin Dublin (−3 Val→Glu): an N-terminal variant which has an aberrant signal peptidase cleavage site

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Antithrombin Dublin is an electrophoretically fast variant of antithrombin which has normal heparin affinity. Direct sequencing of amplified exon 2 revealed a Val→Glu substitution at position −3. N-terminal sequencing of antithrombin from two individuals, heterozygous for the Dublin mutation, showed the presence of a truncated antithrombin in which the N-terminal dipeptide is absent. We propose that the prepeptide mutation redirects signal peptidase cleavage to a site two amino acids downstream into the mature protein.

Antithrombin; N-terminal variant; Truncated serpin

1. INTRODUCTION

Human antithrombin is a 58 kDa heparin-activated serpin and the major inhibitor of thrombin in plasma [1,2]. Its physiological importance is emphasised by the recurrent thromboses experienced by individuals with a deficient or functionally abnormal antithrombin [3]. A number of antithrombin variants having defective thrombin inhibitory activity and, or altered heparin binding have been recognised and been shown to involve mutations primarily in exons 2 and 6 of the coding sequence [4].

Antithrombin Dublin was originally studied in four Irish families with no history of thrombosis and has been the subject of a previous report [5]. It is more negatively charged than normal antithrombin and appears to have normal heparin affinity. In the course of sequencing the antithrombin gene in one antithrombin Dublin heterozygote, we found that the DNA encoding the signal peptide contained a valine to glutamate substitution at position −3 in the signal peptide. A second, unrelated individual, under investigation for recurrent thromboses, was found to have the same mutation. N-Terminal sequence analysis of the variant protein from both individuals indicates the mutation gives rise to an aberrant signal peptidase cleavage site two amino acids downstream of the normal N-terminal histidine occurring after an adjacent glycine.

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2. MATERIALS AND METHODS

2.1. *Materials*

All reagents, unless otherwise stated, were from Sigma. Deoxynucleotides and T4-polynucleotide kinase were obtained from Pharmacia-LKB Biotechnology, *Thermus aquaticus* (Taq) polymerase from Perkin Elmer Cetus and low melting point agarose from Biorad. Ultrapure urea and agarose were from Gibco-BRL. DNA sequencing was performed using Sequenase (Version 2.0, Modified T7 DNA Polymerase) from the United States Biochemical Corporation. Chromogenic substrate TH was obtained from Boehringer Mannheim.

2.2. *Measurement of plasma antithrombin levels*

Antithrombin antigen was measured by competitive ELISA as previously described [6]. Antithrombin anti-IIa activity was measured in the presence of 60 U/l heparin; chromogenic substrate (Chromozym TH) was added after 30 s, and the absorbance change at 405 nm recorded [7]. Anti-Xa activity was measured in the presence of 1000 U/ml heparin using the substrate S2222 as described by Odegaard et al. [8]. Values were expressed as a percentage of a reference plasma defined as 100%.

2.3. *Amplification of exon 2 of the antithrombin gene*

Oligonucleotides 5′-CCAGGTGGGCTGGAATCCTCTGCTTT and 5′-CCATCAGTTGCTGGAGGGTGTCATTA spanning positions 41-507 within segment 2 of the antithrombin sequence [9] were synthesized on an Applied Biosystems DNA Synthesizer and used as amplification primers without further purification. DNA was amplified using the Polymerase Chain Reaction (PCR) as previously described [7]. Following amplification, the 467 bp DNA fragment was purified by electrophoresis in 1% low melting point agarose in TAE. The amplified band was excised under UV-light and the DNA extracted by subjecting the gel slice to two cycles of freezing and thawing [10]. Following centrifugation in a bench top centrifuge, the supernatant was extracted with phenol and chloroform and the DNA recovered by ethanol precipitation. The pellet was washed with 70% ethanol, dried and resuspended in 9 μl H_2O.

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2.4. *Direct sequencing of amplified exon 2*

An oligonucleotide 5'-CTTGGTCATAGCAAAAGCCG spanning positions 451–470 on the non-coding strand of segment 2 of the antithrombin sequence [9] was synthesised as for the amplification primers but purified before use by electrophoresis on a 20% denaturing polyacrylamide gel and subsequent elution. Sequencing was performed using a modified Sanger dideoxy sequencing protocol as described [11].

2.5. *Isolation of antithrombin and N-terminal protein sequencing*

Antithrombin was isolated from plasma by affinity chromatography on heparin Sepharose [12]. The antithrombin fraction was concentrated and dialysed against 20 mM Tris, pH 7.8, and then injected onto a Mono Q HR 5/5 anion exchange column (Pharmacia) attached to a Waters 650E automated chromatography system and equilibrated with 20 mM Tris, pH 7.8. Antithrombin was eluted using a linear gradient of 0 to 1 M NaCl in equilibration buffer over 60 min.

As a final step, the antithrombin was injected onto a C18 reverse phase HPLC column (Waters μBondapak) on a Waters HPLC system equilibrated with 0.1% TFA in water and eluted with a linear gradient to 100% from 0.1% TFA in acetonitrile over 80 min.

The purified antithrombin, that is, the heterozygous mixture of normal and abnormal components, was vacuum dried and N-terminal sequencing performed using an Applied Biosystems automated sequencer.

3. RESULTS

3.1. *Case histories*

Case 1 is a 21-year-old man whose sib was one of the original antithrombin Dublin individuals mentioned in an earlier report [5]. This individual was well and had no history of thrombosis at the time of investigation.

Case 2 is a 42-year-old man who had a coronary thrombosis at age 33 and a spontaneous deep vein thrombosis and pulmonary embolus at age 41.

3.2. *Plasma antithrombin levels*

Plasma antithrombin assays showed both patients had low heparin induced inhibitory activity but normal antigenic levels (Table I).

3.3. *Amplification and sequencing of exon 2*

Amplification of exon 2 in DNA from both individuals generated a single fragment of 467 bp. This fragment encodes amino acid residues Lys-18 to Ala-94 and 77 bp of the 5' flanking intronic sequence. Sequencing of the PCR product revealed two nucleotides at position 189 of segment 2 [9] in the codon for amino acid −3 of the signal peptide (Fig. 1). Thus, the two possible codons are GTG, which codes for the expected Val, or GAG which codes for Glu.

Table I

Plasma antithrombin levels

	Antigenic level (%)	Anti-IIa activity (%)	Anti-Xa activity (%)
Normal Range	80–100	75–127	83–115
Case 1	110	71	82
Case 2	103	74	83

Results are the mean of four determinations. Activity assays were performed in the presence of heparin.

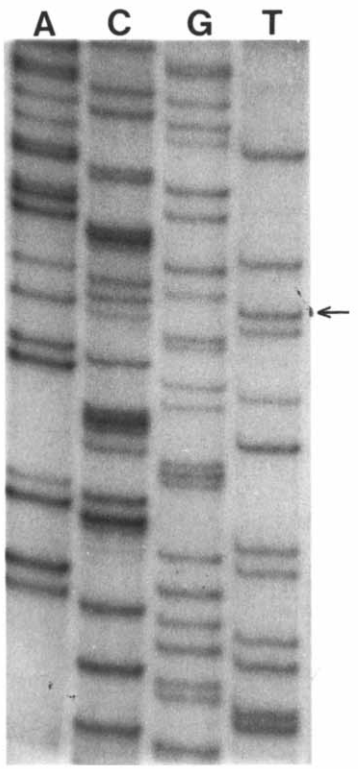

Fig. 1. Direct sequencing of the antisense strand of antithrombin Dublin. The position of the mutation in codon −8 is arrowed. The sequence from the bottom of the gel is complementary to the sense strand; in the normal gene codon −3, which codes for Val, is CAC (GTG on the sense strand) and in antithrombin Dublin codon −3 is CTC (GAG on the sense strand) which codes for Glu.

3.4. *N-Terminal protein sequencing*

In order to examine whether the −3 Val→Glu substitution was associated with an alteration in signal peptide processing, antithrombin was isolated from the plasma of both individuals and subjected to N-terminal sequencing for 7 cycles. Two sequences were found in both samples: H G S P V D I and S P V D I (C) T A. The former corresponds to the expected N-terminal of normal antithrombin whilst the latter is identical apart from the absence of the N-terminal dipeptide, His-Gly. This indicated that the −3 Val→Glu substitution has resulted in aberrant signal peptide cleavage and production of a truncated form of plasma antithrombin beginning at position Ser + 3 (Fig. 2).

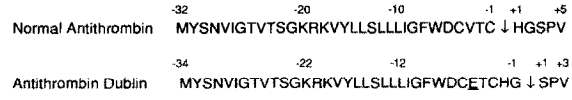

Fig. 2. Predicted amino acid sequence of antithrombin Dublin. Arrows indicate the expected and proposed new site of signal peptidase cleavage. The position of the mutation in antithrombin Dublin is underlined.

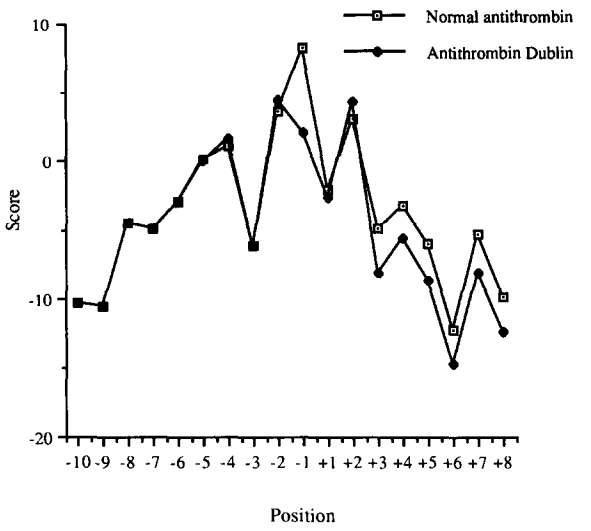

Fig. 3. −13 to +2 weight matrix scores as a function of the position of the moving window. The highest scoring window position is the predicted site of signal peptidase cleavage.

4. DISCUSSION

The Val→Glu substitution in the signal peptide and the appearance of a truncated antithrombin in plasma from the propositi confirms the original description of antithrombin Dublin as an inherited variant with both individuals studied here being heterozygous for the mutation. Antithrombin Dublin is co-eluted with normal antithrombin from heparin Sepharose at a NaCl concentration of 0.7 M suggesting that the N-terminal dipeptide, His-Gly, is not essential for normal heparin affinity. The increased net negative charge of antithrombin Dublin which is observed following isoelectric focusing and immunoblotting of plasma antithrombin is explained by the loss of the positively charged N-terminal His residue. We propose that the negative charge introduced by the −3 Val→Glu mutation redirects signal peptidase cleavage to a new site located two amino acid residues into the mature protein sequence.

Comparisons of aligned amino acid sequences of known prepeptides have revealed some regularities and clues to the specificity of signal peptidase which have been summarised as the '−3, −1' rule [13] or the 'A-X-B' model [14]. Thus, amino acids having small neutral side chains (i.e. Ala, Gly, Ser, Cys, Thr) dominate strongly in positions −1 and −3. Certain hydrophobic residues (i.e. Leu, Lle, Val) are also preferred at position −3 while charged, aromatic and large polar residues are absent from both positions. These qualitative rules have been incorporated by Von Heijne into a method which uses a data base of 161 aligned eukaryotic signal sequences and a standard weight matrix approach to predict the site of signal peptidase cleavage [15]. To locate the most probable cleavage site in antithrombin Dublin, −13 to +2 weight matrices were constructed for the normal and mutated signals using the amino acid counts matrix and the method described by Von Heijne in [15]. The scores obtained by summing the contributions from −13 to +2 were examined as a function of the position of a moving window, the highest scoring window position being the predicted site of cleavage. As expected, the highest score with the normal antithrombin signal was obtained for position −1 (i.e. between Cys−1 and His +1; Score = 8.18 (Fig. 3). With the mutated signal of antithrombin Dublin, the score for position −1 was much smaller than that obtained for the normal sequence, indicating the reduced probability of normal signal peptidase cleavage. The highest scores were obtained for positions −2 (i.e. Thr−2↓Cys−1; Score = 4.51) and +2 (i.e. Gly+2↓Ser +3; Score = 4.29). This indicates that the Val→Glu mutation in antithrombin Dublin generates two alternative sites of signal peptidase cleavage located between Gly−2 and Ser−3, where cleavage occurs, and also between Thr−2 and Cys−1. A relevant analogous example is that of albumin Redhill [16], where, similarly, a prepeptide mutation generates two possible sites of signal peptidase cleavage. Since an extended antithrombin having an N-terminal Cys residue was not detected in plasma from the propositi, we suggest that, in contrast to the case of albumin Redhill [16] Ser is more favoured than Cys in directing signal peptidase cleavage.

The clinical importance of antithrombin Dublin remains unclear. Characterisation of further variants with the identical mutation should clarify the natural history of this variant.

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