AMINO ACID SEQUENCE OF THE FACTOR $XIII_a$ ACCEPTOR SITE IN BOVINE PLASMA FIBRONECTIN

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1. Introduction

Blood coagulation factor XIII is a proenzyme which can be activated by thrombin [1] to the transglutaminase factor $XIII_a$ [2–4]. Factor $XIII_a$ catalyzes the formation of $\epsilon(\gamma$-glutamyl)lysyl amide bonds between pairs of γ-chains in aggregated fibrin, resulting in its transformation to a highly stable and insoluble covalently cross-linked clot (reviewed in [5–7]). Two other plasma proteins α_2-macroglobulin and fibronectin contain acceptor sites for factor $XIII_a$ as shown by incorporation of dansylcadaverine [8]. Only fibronectin, but not α_2-macroglobulin, was shown to be cross-linked to fibrin [8]. Cross-linking of fibronectin to collagen [9,10] and to *Staphyloccocus aureus* cells [11] has been demonstrated. It has also been suggested that α_2-antiplasmin could be covalently linked to fibrin in a Ca^{2+}-dependent reaction probably catalyzed by factor $XIII_a$ [12].

Here we report the amino acid sequence in bovine fibronectin which contains the glutamine residue labelled with radioactive putrescine by factor $XIII_a$. This glutamine is located at position 3 from the *N*-terminus of fibronectin.

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2. Materials and methods

Bovine plasma fibronectin was purified by affinity chromatography on gelatin agarose essentially as described for human plasma fibronectin [13]. Bovine thrombin [14], human factor XIII [15] and human plasminogen [16] were purified as described.

In the labelling experiment 1.7 g fibronectin was dissolved in 2.6 litres 50 mM Tris, 0.15 M NaCl, 10 mM $CaCl_2$ (pH 7.5) and 418 mg putrescine/2 HCl (Sigma) containing 250 μCi [1,4-^{14}C]putrescine/2 HCl (116 mCi/mmol, Amersham) was added. Factor XIII, 4 ml was activated for 1 h at 37°C with 500 units thrombin (1 ml factor $XIII_a$ incorporated 25 000 cpm [^{14}C]-putrescine into casein under non-reducing conditions) and added to the fibronectin/putrescine solution. The reaction mixture was incubated at 37°C overnight and dialyzed for 24 h against 25 litres 0.1 M NH_4HCO_3 (pH 8.3).

Plasminogen (50 mg) was activated with 1 mg streptokinase (Kabi) in 100 ml 0.2 M Tris (pH 8.4) containing 10 mM lysine and added to the solution of putrescine labelled fibronectin. After incubation for 20 h at 37°C the digestion was arrested with 20 mg Aprotinin (Novo). The resulting fragments were separated on columns of gelatin agarose (5 × 25 cm) and diethylaminoethyl cellulose (DE-52, Whatman) 5 × 15 cm both equilibrated in 0.1 M NH_4HCO_3 (pH 8.3) and coupled in a tandem arrangement with the gelatin agarose as the first one. The solution was pumped

(120 ml/h) through the columns followed by washing with 1 litre 0.1 M NH_4HCO_3. The columns were disconnected and the gelatin–agarose eluted with a linear gradient formed from 1 litre 0.1 M NH_4HCO_3 and 1 litre 0.1 M NH_4HCO_3, 5 M urea (pH 8.3). The DE-52 column was eluted with a linear gradient in NH_4HCO_3 (pH 8.3) (0.5 litre 0.1 M NH_4HCO_3 and 0.5 litre 0.6 M NH_4HCO_3). Radioactivity was monitored by scintillation counting on 20 μl aliquots of the effluents.

Reduction and alkylation with iodoacetic acid of the M_r 29 000 fragment, digestion with trypsin and thermolysin, peptide separation on DEAE-Sephacel, high-voltage paper electrophoresis at pH 6.5 and 2.1, fingerprinting, autoradiography, sequencing and amino acid analysis were performed essentially as in [17]. Putrescine was eluted from the amino acid analyzer column after 134 min by extended use of the last buffer. Peptides were derivatised for mass spectrometric measurements according to standard procedures [18,19]. Spectra were recorded on a Kratos MS50 high-field magnet mass spectrometer operating with an accelerator voltage of 8 kV and an electron beam energy of 70 eV.

3. Results and discussion

Plasma fibronectin is a glycoprotein composed of 2 disulphide bridged chains with M_r 220 000 and 215 000 [20]. Fragmentation of fibronectin with proteolytic enzymes and chemicals has been intensively studied [21–27] and after digestion of [^{14}C]putrescine labelled fibronectin with plasmin the radioactivity was located in a fragment with M_r 27 000 [28].

To determine the amino acid sequence around the acceptor site in fibronectin, purified bovine fibronectin was incubated with [^{14}C]putrescine and purified human factor $XIII_a$ under non-reducing conditions. The labelled fibronectin was digested with plasmin and the resulting fragments separated on columns of gelatin–agarose and DEAE-cellulose. Four fragments with M_r 29 000, 170 000, 23 000 and 6000 had been isolated after digestion of unlabelled bovine fibronectin with plasmin [29]. Similar fragments were isolated from the labelled fibronectin. The M_r 29 000 fragment, which did not absorb to either of the two columns, contained all the radioactivity, while the M_r 170 000 eluted from the gelatin agarose and the M_r 23 000 and 6000 fragments eluted from the DEAE-cellulose were free of any detectable radioactivity. The M_r 29 000 fragment was reduced, alkylated and digested with trypsin. The resulting peptides were separated on DEAE–Sephacel in a gradient of NH_4HCO_3 followed by paper electrophoresis at pH 6.5 and 2.1. The amino acid composition of the main radioactive peptide (T1) is shown in table 1. No sequence information was obtained either by direct Edman degradation or by dansylation of T1. The complete amino acid sequence of the M_r 29 000 fragment has been determined (in preparation) and the amino acid composition of T1 corresponded to the N-terminal 36 residues (fig.1) of this fragment. The values for proline and glycine are somewhat high when compared with the sequence due to trace peptide impurities originating from the gelatin column. T1 was further digested with thermolysin and the resulting peptides separated by two-dimensional paper electrophoresis at pH 6.5 and 2.1. Peptides corresponding to residues 1–4, 5–11, 12–13, 28–29 and 30–36 were obtained in pure form as judged from amino acid compositions and sequencing results. Peptide material derived from residues 14–27 did not show up after staining with fluorescamine and no further effort was made to locate such material because the only radioactive spot detected by autoradiography coincided with the posi-

Table 1
Amino acid composition of the peptide labelled with putrescine by factor $XIII_a$ and isolated after digestion with trypsin

		a
Cmc	0.4	1
Asx	3.1	3
Thr	1.0	1
Ser	2.7	3
Glx	9.2	10
Pro	4.3	3
Gly	3.0	2
Ala	1.2	1
Val	2.0	2
Met	0.0	0
Ile	1.7	2
Leu	1.1	1
Tyr	2.0	2
Phe	0.0	0
Lys	2.1	2
His	1.0	1
Trp	n.d.	1
Arg	1.0	1
Putrescine	0.9	

[a] From the sequence

1 ↓ 5 10 15 20

<Glu-Ala-Gln-Gln-Ile-Val-Gln-Pro-Gln-Ser-Pro-Leu-Thr-Val-Ser-Gln-Cys-Lys-Pro-Gly-

25 30 35

-Ser-Tyr-Asp-Asn-Gly-Lys-His-Tyr-Gln-Ile-Asn-Gln-Gln-Trp-Glu-Arg-

Fig.1. The N-terminal 36 residues of bovine plasma fibronectin. The (↓) indicates the glutamine residue to where putrescine is linked by factor $XIII_a$.

tion of peptide 1–4. The amino acid composition of this peptide was Glx 3.5, Ala 1.0, putrescine 0.9. After deuteroacetylation and permethylation of this peptide the electron impact mass spectrum shown in fig.2 was obtained. This spectrum allows the assignment of the sequence as:

<Glu–Ala–Glx–
|
Putrescine

from the signals at *m/e* 98, 211, 469 and 497 as indicated in fig.2. For comparison the same peptide was isolated from unlabelled fibronectin and after deuteroacetylation and permethylation the electron-impact mass spectrum obtained is shown in fig.3. In agreement with the expected sequence:

<Glu–Ala–Gln–

the signals at *m/e* 98, 211, 353 and 381 were found. Immonium ion fragments ($H_3C–\overset{+}{N}H=CH–R$) are observed at *m/e* 259 ($R = (CH_2)_2CO–N(CH_3)–(CH_2)_4–N(CH_3)–CO–C^2H_3$) and *m/e* 143 ($R = (CH_2)_2CO–N(CH_3)_2$) in fig.2 and 3, respectively. Neither of the two peptides yielded a molecular ion.

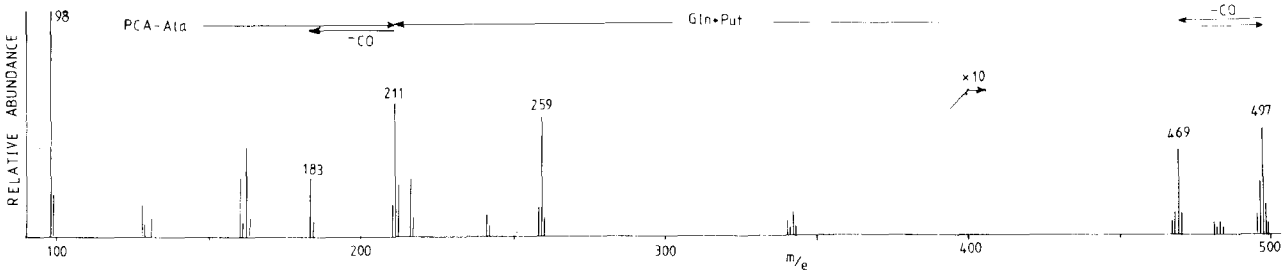

Fig.2. The electron-impact mass spectrum obtained at 260°C of the deuteroacetylated permethylated peptide (residues 1–4) containing the putrescine label.

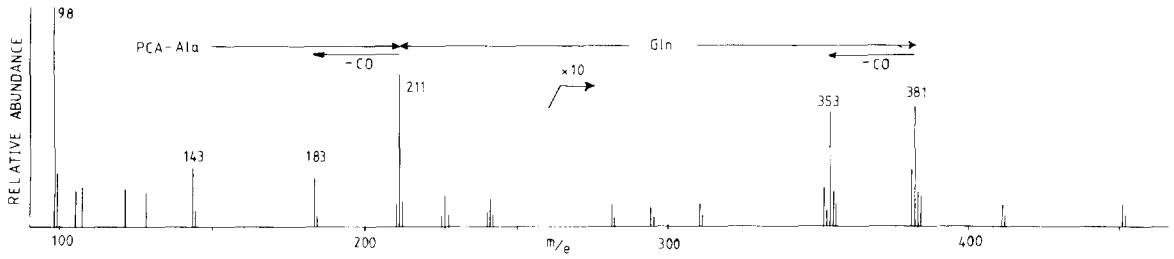

Fig.3. The electron-impact mass spectrum obtained at 260°C of the unlabelled deuteroacetylated permethylated peptide (residues 1–4).

This is probably due to a rapid cyclisation and cleavage of the C-terminal Gln-4 resulting in pyroglutamic acid fragments.

Two other radioactive peptides were detected in the effluent from the DEAE–Sephacel column and after further purification by high-voltage paper electrophoresis at pH 6.5 and 2.1 shown to correspond to positions 1–12 and 1–26, respectively, in fig.1. No N-terminus was found by dansylation or Edman degradation. After digestion with thermolysin followed by two-dimensional fingerprinting at pH 6.5 and 2.1 both peptides gave rise to the same radioactive peptide corresponding to positions 1–4 in fig.1.

Our results show that putrescine is linked to the Gln residue located at position 3 in bovine fibronectin, and no indication for incorporation of putrescine into the Gln residue at position 4, other of the Gln residues at positions 7, 9, 16, 29, 32 and 33 or any other positions in fibronectin has been found. The labelling was performed under non-reducing conditions in order to keep the fibronectin as native as possible. Whether a reducing agent in the reaction mixture will result in the labelling of other positions in fibronectin was not investigated.

The first 6 residues in human fibronectin have been determined [26] as:

<Glu–Ala–Glx–Glx–Met–Val–

If the Glx residue in position 3 is a glutamine it will very likely be a substrate site for factor $XIII_a$.

In fig.4 the acceptor sites for factor $XIII_a$ in human fibrinogen [30,31] bovine β-casein [32] and bovine fibronectin have been aligned. No sequence homology is obvious. As in fibronectin it is the first of 2 consecutive glutamine residues in the γ-chain of fibrinogen which is labelled [30].

The abnormal scar formation in patients deficient in factor XIII [33,34], the effect of factor XIII on the growth of fibroblasts in cell culture [34] and the in vitro cross-linking of fibronectin to fibrin and collagen by factor $XIII_a$ [8–10] indicate that ε(γ-glutamyl)lysyl amide bonds are of physiological importance not only between γ-chains of fibrin but more generally in the matrix of connective tissue. The identification of a potential cross-linking site in plasma fibronectin should facilitate the isolation and characterization of cross-links formed in vivo to fibronectin.

The requirement for fibronectin in the opsonization of foreign particulate matter [36] by the reticuloendothelial system and the suggested involvement of transglutaminases in receptor-mediated endocytosis [37] raise the question whether the Gln-residue in position 3 in fibronectin have a specific function in connection with the transport of fibronectin-coated particles through membranes.

Acknowledgements

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                                                    397
fibrinogen γ-chain human          -Thr-Ile-Gly-Glu-Gly-Gln-Gln-His-His-Leu-Gly-

                                                    328
fibrinogen α-chain human          -Gly-Ser-Thr-Gly-Asn-Gln-Asn-Pro-Gly-Ser-Pro-

                                                    366
fibrinogen α-chain human          -Ser-Gly-Ser-Thr-Gly-Gln-Trp-His-Ser-Glu-Ser-

                                                    167
β-casein bovine                   -Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-

                                                      3
plasma fibronectin bovine                 <Glu-Ala-Gln-Gln-Ile-Val-Gln-Pro-
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Fig.4. Alignment of the factor $XIII_a$ acceptor sites in fibrinogen [30,31] β-casein [32,35] and plasma fibronectin. The numbers indicate the positions of the sites in the respective chains.

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