Isolation of a fibrin-binding fragment from blood coagulation Factor XIII capable of cross-linking fibrin(ogen)

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Purified platelet Factor XIII was radioiodinated and then partially degraded by thrombin or trypsin, and a fibrin-binding fragment was identified by autoradiography and immunoblotting following separation by SDS/polyacrylamide-gel electrophoresis. Limited proteolysis of ^{125}I -Factor XIII by thrombin or trypsin produced an ^{125}I -51 kDa fragment and an unlabelled 19 kDa fragment. The 51 kDa fragment was purified by h.p.l.c. on a TSK-125 gel-filtration column. Partial amino acid sequence analysis of the 51 kDa fragment indicated that it was similar in sequence to the Gly³⁸–Lys⁵¹³ segment in placental Factor XIII a-chain. More than 70 % of the 51 kDa fragment bound to fibrin, whereas the 19 kDa fragment did not bind. The active site was localized to the 51 kDa fragment since this fragment expressed transglutaminase activity, cross-linked fibrin and fibrinogen and incorporated iodo[^{14}C]acetamide into the active-site cysteine residue. Isolation of a fibrin-binding fragment expressing transglutaminase activity demonstrates that each a-chain of the dimeric Factor XIIIa could function independently to cross-link fibrin. The fibrin-binding site could play an important role in localizing Factor XIIIa to the fibrin clot.

INTRODUCTION

Blood coagulation Factor XIII occurs as a zymogen in plasma, platelets and the placenta (McDonagh, 1987). Platelet and placental Factor XIII are composed of two identical 80 kDa polypeptides (a-chains), whereas plasma Factor XIII is composed of two a-chains that are biochemically and immunologically indistinguishable from platelet Factor XIII a-chains, and two 75 kDa b-chains, forming a tetramer (McDonagh, 1987). The nucleotide sequences of the genes and amino acid sequences of the a-chains of platelet and placental Factor XIII have been published and are similar (Ichinose *et al.*, 1986; Takahashi *et al.*, 1986; Grundmann *et al.*, 1986).

During haemostasis the cleavage of a 4 kDa activation peptide from the a-chains of the plasma Factor XIII by thrombin results in the formation of an inactive intermediate, Factor XIII' (Curtis *et al.*, 1974). Cleavage of the activation peptide occurs between residues Arg-37 and Gly-38 (Takagi & Doolittle, 1974; Takahashi *et al.*, 1986) near the N-terminus of the a-chains. The binding of fibrinogen to Factor XIII' at physiological Ca^{2+} concentration promotes the dissociation of the b-chains (Credo *et al.*, 1978) and results in the formation of the active enzyme, Factor XIIIa. Factor XIIIa (a transglutaminase) rapidly catalyses the formation of γ -glutamyl- ϵ -lysyl covalent bonds between the α - and γ -chains of polymerized fibrin molecules (McDonagh, 1987).

Soluble fibrinogen, polymerizing fibrin monomers and insoluble fibrin fibres specifically bind the a-chains of plasma and platelet Factor XIII (Greenberg & Shuman, 1982; Greenberg *et al.*, 1985). The b-chains do not bind to fibrin and do not interfere with a-chain binding (Greenberg *et al.*, 1985). When blood clots are formed *in vitro* the concentration of free plasma Factor XIII is

decreased by over 90 % (Lorand & Dickerman, 1955; Divlansky *et al.*, 1970). In addition, the binding of plasma Factor XIII to fibrin greatly decreases the thrombin concentration required for cleavage of the activation peptide (Janus *et al.*, 1983; Lewis *et al.*, 1985; Greenberg & Miraglia, 1985; Greenberg *et al.*, 1987). These data suggested the presence of a fibrin-binding domain in the a-chains of Factor XIII that regulates the localization of Factor XIIIa to the fibrin clot and Factor XIIIa formation during haemostasis (Credo *et al.*, 1981; Lorand, 1986; Greenberg *et al.*, 1987).

The purpose of the present study was to isolate the fibrin-binding domain from the a-chain of Factor XIII. A 51 kDa fragment was formed after cleavage of the 4 kDa activation peptide from the N-terminus and a 19 kDa fragment from the C-terminus. The 51 kDa fragment specifically bound to fibrin, expressed transglutaminase activity and cross-linked fibrinogen and fibrin. These results have important implications for understanding the mechanism of Factor XIIIa cross-linking of fibrin.

MATERIALS AND METHODS

Protein purification

Platelet Factor XIII was purified from outdated human platelets as described previously (Schwartz *et al.*, 1971). Human fibrinogen grade L was purchased from Kabi Diagnostica (Stockholm, Sweden) and purified as described previously (Greenberg & Shuman, 1982). The purity of the proteins was evaluated by SDS/PAGE (Laemmli, 1970). Electrophoresis samples were always reduced by boiling for 5 min in the presence of 1 % (v/v) 2-mercaptoethanol before electrophoresis except for the samples destined for protein sequence analysis.

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; TBS, Tris-buffered saline (130 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.4).

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Measurement of Factor XIIIa activity

Purified platelet Factor XIIIa or purified tryptic fragments of platelet Factor XIII were assayed for transglutaminase activity by the [^3H]putrescine-incorporation assay (Miraglia & Greenberg, 1985). Human α -thrombin (8160 NIH units/ml; 72.6 μM) supplied by Dr. J. W. Fenton, II (New York State Department of Health, Albany, NY, U.S.A.), was used for activating Factor XIII (Miraglia & Greenberg, 1985). Specific activity of purified platelet Factor XIIIa or purified 51 kDa peptide is expressed as nmol of putrescine incorporated into casein by 1 mg of protein in 1 h at 37 °C.

Radioiodination of platelet Factor XIII

Purified platelet Factor XIII a-chains were radio-labelled as previously described (Greenberg & Shuman, 1982). The autoradiogram of ^{125}I -Factor XIII separated by SDS/PAGE was quantified with a GS 300 scanning densitometer (Hoefer Scientific, San Francisco, CA, U.S.A.). Radiolabelled platelet Factor XIII migrated as an 80 kDa band containing over 96% of the label. The specific radioactivity of ^{125}I -Factor XIII varied between 3800 and 5500 c.p.m./ng. Radioiodinated Factor XIIIa cross-linked fibrin at the same rate as native platelet Factor XIIIa.

Immunoblotting

Immunoblotting was performed according to the procedure of Towbin *et al.* (1979). In this procedure proteins separated by SDS/PAGE were electrophoretically transferred to nitrocellulose paper (Bio-Rad Laboratories, Richmond, CA, U.S.A.) by using a Hoefer Scientific blotting apparatus at 0.5 A for 5 h. The nitrocellulose was then incubated with 1% (w/v) bovine serum albumin in TBS to saturate residual protein-binding sites. To identify Factor XIII a-chains, the nitrocellulose was then incubated sequentially with rabbit anti-(Factor XIII a-chain) serum (Calbiochem, La Jolla, CA, U.S.A.) for 12 h at 4 °C and anti-(rabbit IgG) antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories) for 4 h at 4 °C. Factor XIII a-chain antigen was detected by immersing the immunoblots in a colour development reagent (Bio-Rad Laboratories).

Limited proteolysis of Factor XIII

To identify proteolytic fragments of Factor XIII, a mixture of radio-labelled (1×10^5 c.p.m.) and unlabelled platelet Factor XIII (5–10 μg) was digested with either thrombin or trypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.). Reactions were carried out in TBS containing 5 mM- CaCl_2 or 5 mM-EDTA respectively at 37 °C for 30 min. The α -thrombin concentrations ranged from 10 to 100 NIH units/ml. In the trypsin reactions a 100:1 (w/w) Factor XIII/trypsin ratio was used. Proteolysis was stopped by the addition of solubilizing buffer (4% SDS/6 M-urea/20 mM-EDTA/12.5 mM-Tris/HCl buffer, pH 6.8), and the reaction mixtures were subjected to SDS/PAGE and immunoblotting. The nitrocellulose paper was then exposed to Kodak X-OMAT XAR-5 film placed between Dupont Cronex enhancing screens for 9 h at -70 °C. The X-ray film was developed and the bands were aligned with the Factor XIII-related antigens appearing on the immunoblots. Band intensities on the X-ray film were quantified by scanning densitometry.

Active-site modification of Factor XIII with iodo[^{14}C]acetamide

Platelet Factor XIII (30 $\mu\text{g}/\text{ml}$) was digested with trypsin (0.3 $\mu\text{g}/\text{ml}$) in TBS containing 5 mM- CaCl_2 for 30 min at 37 °C. Trypsin proteolysis was inhibited by the addition of soya-bean trypsin inhibitor (1 $\mu\text{g}/\text{ml}$) (Sigma Chemical Co., St. Louis, MO, U.S.A.). Iodo[^{14}C]acetamide (New England Nuclear, Boston, MA, U.S.A.; 20 μCi) was incubated with either Factor XIIIa or trypsin-treated Factor XIII for 30 min at 37 °C. Following SDS/PAGE the proteins were detected by staining with Coomassie Brilliant Blue or by immunoblotting. The fragment containing the active site was identified by autoradiography of the dried gel or nitrocellulose paper.

Fibrin-binding assay of Factor XIII

The binding of Factor XIII to fibrin was carried out as described previously (Greenberg *et al.*, 1985). Briefly, portions of ^{125}I -labelled platelet Factor XIII (1×10^5 c.p.m.) and unlabelled platelet Factor XIII (50–100 $\mu\text{g}/\text{ml}$) were mixed and incubated with fibrinogen (0.50 mg/ml), thrombin (1 NIH unit/ml) and 5 mM- CaCl_2 in TBS for 30 min at 37 °C. Fibrin was then spun down (Beckman model B Microfuge) and washed thrice by resuspension in TBS followed by centrifugation. Radioactivity associated with the fibrin pellet was measured in a γ -radiation spectrometer and found to represent 40–50% of the total label. Further dissociation of the label from the fibrin did not occur even after 18 h of incubation at 37 °C. The washed sonicated fibrin containing bound Factor XIII was then solubilized, reduced in 1% 2-mercaptoethanol and subjected to SDS/PAGE, immunoblotting and autoradiography. The amount of Factor XIII bound was also quantified by scanning densitometry of the radioactive Factor XIII band on the X-ray film.

Identification of a fibrin-binding fragment in Factor XIII by SDS/PAGE and immunoblotting

A mixture of ^{125}I -Factor XIII (10^5 – 10^6 c.p.m.) and platelet Factor XIII (5–50 μg) was digested with either thrombin in the presence of 5 mM-EDTA or trypsin in the presence of 5 mM- CaCl_2 as described above under 'Limited proteolysis of Factor XIII'. The reactions were stopped by using a final concentration of 10 μM -phenylmethanesulphonyl fluoride and soya-bean trypsin inhibitor. The proteolysed Factor XIII fragments were mixed with fibrinogen (0.5 mg/ml), 5 mM- CaCl_2 and 10 units of thrombin/ml to clot the fibrinogen. The fibrin containing bound Factor XIII fragments was spun down and washed with TBS to remove unbound fragments. Radioactivity from the fibrin-bound Factor XIII fragments was measured in a γ -radiation counter. The fibrin-bound fragments of Factor XIII were dissolved in solubilizing buffer, electrophoresed and stained with Coomassie Blue. The destained gels were dried and analysed by autoradiography. Identical reaction mixtures were also analysed by SDS/PAGE and immunoblotting. Molecular-mass standards (High/Low from Bio-Rad Laboratories and prestained molecular-mass markers from Sigma Chemical Co.) were always included, and ^{14}C -labelled protein molecular-mass standards (Amersham Corp., Arlington Heights, IL, U.S.A.) were used additionally for calculation of molecular masses of peptides identified by autoradiography.

Isolation of a 51 kDa fibrin-binding fragment from a partial trypsin digest of platelet Factor XIII

A mixture of ^{125}I -Factor XIII (1×10^5 c.p.m.) and unlabelled Factor XIII (25 μg) was incubated with trypsin (0.25 μg) and 5 mM- CaCl_2 in TBS for 30 min at 37 °C. The reaction was stopped by the addition of 200 μM -*p*-amidinophenylmethanesulphonyl fluoride (Calbiochem), and the partial trypsin digest was separated by gel-filtration h.p.l.c. on a Bio-Sil TSK-125 column (Bio-Rad Laboratories). The column was equilibrated and eluted with 50 mM- Na_2SO_4 /20 mM- NaH_2PO_4 buffer, pH 6.8, at a flow rate of 1.0 ml/min. Molecular-mass standards (Bio-Rad Laboratories) were used to calibrate the columns. The protein peaks emerging from the h.p.l.c. column were analysed for purity by SDS/PAGE under reducing conditions.

Specificity of binding of the 51 kDa polypeptide to fibrin

Binding of the ^{125}I -51 kDa fragment and ^{125}I -Factor XIII to fibrin was performed as described above (see Greenberg *et al.*, 1985) with the following exception: TBS buffer containing 1 mg of bovine serum albumin/ml was used to block non-specific fibrin-binding sites. Binding of the labelled 51 kDa fragment was competitively inhibited with a 100-fold molar excess of purified platelet Factor XIII. In some experiments iodoacetamide (10 mM) or 6-aminohexanoic acid (100 mM) was also added to the binding mixture.

Cross-linking of fibrinogen or fibrin by the 51 kDa polypeptide

Fibrinogen (0.5 mg/ml) or fibrin monomers (0.5 mg/ml) were incubated with the 51 kDa fragment (0.01 mg/ml) or Factor XIIIa (0.01 mg/ml) at 37 °C in TBS buffer containing either 5 mM- CaCl_2 or 5 mM-EDTA. The reaction was stopped after 10 min (with Factor XIIIa) or 30 min (with 51 kDa fragment) by adding 2% SDS, 6 M-urea and 5% (v/v) 2-mercaptoethanol, the mixtures were boiled and the samples were separated by SDS/PAGE. Gels were stained with Coomassie Blue and the extent of cross-linking was quantified by scanning densitometry.

Amino acid sequence determination

A 870 μg portion of the trypsin-digested platelet Factor XIII was subjected to SDS/PAGE. The 51 kDa and 19 kDa fragments of this digest were recovered from the gel by electrophoretic elution (Hunkapiller *et al.*, 1983), by using the electrophoresis tank and elution cell purchased from C.B.S. Scientific (Del Mar, CA, U.S.A.).

The N-terminal sequence of the 51 kDa and 19 kDa fragments was established by automated Edman degradation in an Applied Biosystems (Foster City, CA, U.S.A.) 470 A gas/liquid-phase sequencer with the O2RPTH program and chemicals supplied by the manufacturer. The phenylthiohydantoin derivatives were analysed immediately after each cycle by reverse-phase h.p.l.c. on a Hewlett-Packard 1090 liquid chromatograph with an ABI PTH- C_{18} 2.1 mm $\times$ 220 mm cartridge and the conditions recommended by the manufacturer.

The purified 51 kDa fragment was further proteolysed by *Staphylococcus aureus* V8 proteinase, reduced, alkylated and separated by h.p.l.c. as described previously (Weisberg *et al.*, 1986). The h.p.l.c.-purified peptides

were subjected to amino acid sequence analysis in a gas-phase sequencer from Applied Biosystems.

RESULTS

Identification of radiolabelled and unlabelled fragments after limited proteolysis of Factor XIII

The fragments from partial proteolysis of ^{125}I -labelled Factor XIII a-chains with thrombin and trypsin were analysed by SDS/PAGE, autoradiography and immunoblotting to determine the extent of proteolysis, radioiodination and reactivity toward anti-(Factor XIII) serum. By autoradiography, the thrombin (100 units/ml) digest revealed two fragments with molecular masses 76 kDa (Factor XIIIa) and 51 kDa (Fig. 1, lane 6). Thrombin at lower concentrations (10 units/ml) resulted only in the formation of Factor XIIIa (76 kDa) (Fig. 1, lane 5). The Factor XIII fragments separated by SDS/PAGE were treated with anti-(Factor XIII a-chain) antiserum to detect unlabelled proteolytic fragments. Through this technique, an unlabelled 19 kDa fragment was identified in the digest with 100 units of thrombin/ml (Fig. 1, lane 3). We cannot exclude the possibility that a small amount of radioactivity insufficient for detection was incorporated into the 19 kDa peptide. Similar results were obtained from two different preparations of platelet Factor XIII.

Partial proteolysis of the a-chains by trypsin was also examined to determine whether the proteolysis pattern was similar to that of thrombin. An unlabelled 19 kDa peptide was formed by trypsin proteolysis in addition to a radiolabelled 51 kDa fragment (Fig. 2, lanes 2 and 3). In some experiments two fragments, 55 and 51 kDa, were evident, suggesting an association of the 4 kDa (activation) peptide with the 51 kDa fragment (Fig. 2, lane 2).

Fig. 1. Thrombin proteolysis of ^{125}I -Factor XIII

Platelet Factor XIII (15 μg , 1×10^5 c.p.m.) in the presence of 5 mM-EDTA was incubated with 10 units of thrombin/ml (lanes 2 and 5) or 100 units of thrombin/ml (lanes 3 and 6) at 37 °C for 30 min. Thrombin was inhibited, and the samples were separated by SDS/PAGE and subjected to immunoblotting (lanes 1–3) and autoradiography (lanes 4–6). A radiolabelled 51 kDa fragment and an unlabelled 19 kDa peptide were identified. Lanes 1 and 4 depict intact Factor XIII. A small amount of radiolabelled Factor XIIIa can be seen in the autoradiogram (lane 6) but not in the immunoblot (lane 3).

Fig. 2. Trypsin proteolysis of platelet Factor XIII

Platelet Factor XIII (15 μ g, 1×10^5 c.p.m.) was incubated with trypsin (0.15 μ g) in the presence of 5 mM- CaCl_2 at 37 °C for 30 min. The fragments were separated by SDS/PAGE and analysed by autoradiography (lane 3) and immunoblotting (lane 2). Lane 1 represents intact Factor XIII.

Identification and purification of the fibrin-binding fragment in Factor XIII a-chains

When ^{125}I -Factor XIII was digested by thrombin or trypsin and the fragment bound to fibrin was analysed by autoradiography, only a radiolabelled 51 kDa fragment was detected (results not shown). Since the 19 kDa fragment was not radiolabelled (Fig. 1), Factor XIII fragments bound to fibrin were also analysed by immunoblotting. By the use of this technique the selective binding of the 51 kDa fragment to fibrin could be demonstrated (Fig. 3, lane 5). Although both 51 kDa and 19 kDa fragments were detected in the trypsin digest (Fig. 3, lane 3), the 19 kDa fragment did not bind to fibrin (Fig. 3, lane 5) and remained in the supernatant (Fig. 3, lane 4). The 51 kDa fragment from the thrombin digest of Factor XIII also bound to fibrin, but not the 19 kDa fragment (results not shown). A partial trypsin digest of radiolabelled Factor XIII was fractionated by gel-filtration h.p.l.c. (Fig. 4b). Fractions from the column eluate corresponding to peak II (molecular mass 51 kDa by h.p.l.c. and by SDS/PAGE under reducing conditions) were pooled from five separate experiments. The pooled fractions were concentrated and rechromatographed on the h.p.l.c. gel-filtration column, yielding one major protein peak with a molecular mass of 51 kDa (Fig. 4c). This Factor XIII fragment migrated in SDS/PAGE under both reducing and non-reducing conditions as a single protein band with the same molecular mass (51 kDa) (Fig. 4c inset). Purified platelet Factor XIII was eluted from the h.p.l.c. gel-filtration column as a dimer with a molecular mass under non-reducing conditions of approx. 160 kDa (Fig. 4a) and migrated as a single band on SDS/PAGE under reducing conditions with a molecular mass of 80 kDa (Fig. 4a inset). There was a

Fig. 3. Binding of the 51 kDa fragment to fibrin

Purified platelet Factor XIII (10 μ g) was proteolysed by trypsin (0.1 μ g) in TBS containing 5 mM- CaCl_2 at 37 °C for 30 min. Trypsin was inhibited, and the fragments were incubated with fibrinogen (0.5 mg/ml) and thrombin (1 NIH unit/ml). The fibrin was collected, washed and analysed by SDS/PAGE and immunoblotting. Lane 1, Factor XIII; lane 2, Factor XIIIa; lane 3, trypsin digest of Factor XIII; lane 4, unbound tryptic fragments in the supernatant; lane 5, 51 kDa fragment bound to fibrin. Similar results were obtained when radiolabelled Factor XIII was digested by trypsin or thrombin, bound to fibrin and the binding analysed by autoradiography.

minor 30 kDa contaminant (Fig. 4a) that represented approx. 20% of the radiolabelled material. However, this peptide was not a factor XIII-related antigen since it failed to react with anti-(Factor XIII) antibody (results not shown). Only pooled peak fractions of Factor XIII (peak I, Fig. 4a) were taken for subsequent analyses.

Binding of Factor XIII or 51 kDa fragment to fibrin

Factor XIII or the 51 kDa proteolytic fragment (from trypsin or thrombin digests) bound to fibrin to the same extent, 41.4% and 42.6% respectively. Increasing the amount of fibrinogen from 0.5 mg/ml to 2 mg/ml caused an additional 31% and 34% binding to fibrin of the unbound Factor XIII and 51 kDa fragment respectively (total binding 75–80%). The binding of the 51 kDa fragment to fibrin was inhibited by 48% by a 100-fold molar excess of platelet Factor XIII but not by albumin. Limited quantities of the purified 51 kDa fragment made it difficult to compare further the relative affinities of the 51 kDa fragment and native Factor XIII to fibrin. The presence of 100 mM-6-aminohexanoic acid or modification of the active-site cysteine thiol group by 10 mM-iodoacetamide did not inhibit the binding of Factor XIIIa or the 51 kDa fragment to fibrin.

Localization of the active site to the 51 kDa fragment

The specific activities of platelet Factor XIIIa and the purified 51 kDa fragment were compared by using the [^3H]putrescine-incorporation assay. The transglutaminase activity of Factor XIIIa was 1400 nmol of putrescine

Fig. 4. Purification of the 51 kDa fibrin-binding polypeptide from a partial trypsin digest of platelet Factor XIII

(a) H.p.l.c. gel-filtration profile and SDS/PAGE of ^{125}I -labelled platelet Factor XIII. (b) H.p.l.c. profile and SDS/PAGE of trypsin-treated ^{125}I -Factor XIII. (c) H.p.l.c. profile and SDS/PAGE of purified 51 kDa fragment; peak II represents the 51 kDa fragment. The numbers 640, 158, 44 and 17 refer to the molecular masses (in kDa) of thyroglobulin, γ -globulin, ovalbumin and myoglobin respectively, which were used to calibrate the h.p.l.c. column. The SDS/PAGE profile of the ^{125}I -proteins and fragments is shown in the inset.

incorporated/min per mg compared with an activity of 482 nmol of putrescine incorporated/min per mg for the 51 kDa fragment. In addition, the 51 kDa polypeptide expressed transglutaminase activity even without thrombin treatment. We also found that iodo[^{14}C]acetamide was incorporated into Factor XIIIa (76 kDa) and into the 51 kDa fragment, providing further evidence that the active-site cysteine residue resides in this peptide. Finally, both intact Factor XIIIa and the 51 kDa fragment could cross-link the γ - and α -chains of fibrinogen, forming γ - γ dimers and α -chain multimers (Fig. 5). The rate of Factor XIIIa cross-linking was nearly 3-fold faster than that of the 51 kDa fragment (Fig. 5). However, on a molar basis the 51 kDa fragment had only 22% of the transglutaminase activity of Factor XIIIa (see above).

Fig. 5. Cross-linking of fibrinogen by either purified Factor XIIIa (lane 2) or purified 51 kDa fragment (lane 3)

Identical results were obtained when fibrin monomers were substituted for fibrinogen. The cross-linking by Factor XIIIa (lane 2) was carried out for 10 min, and cross-linking by 51 kDa fragment (Lane 3) was allowed to proceed for 30 min. Lane 1 depicts the Coomassie Blue stain pattern of fibrinogen. When the cross-linking reactions were carried out in the presence of 5 mM-EDTA, the pattern was identical with that seen in lane 1. The notation [α] n denotes cross-linked α -chain multimers. For more details see the text.

N-Terminal amino acid sequence analysis of the fibrin-binding fragments

Automated Edman degradation of about 200 pmol of the 51 kDa and the 19 kDa fragments (Table 1) allowed these fragments to be located in the amino acid sequence of the α -chain of blood coagulation Factor XIII (Takahashi *et al.*, 1986; Grundmann *et al.*, 1986; Ichinose *et al.*, 1986). The 51 kDa fibrin-binding fragment started at position Gly 38 , the N-terminal of the thrombin-activated enzyme. The 19 kDa fragment started at Ser 514 , the trypsin-cleavage 'inactivation site' identified by Takahashi *et al.* (1986).

Amino acid sequence analysis of a peptide derived from the *Staphylococcus*-V8-proteinase digest of the

Table 1. Amino acid sequence data of 51 kDa and 19 kDa fragments

The positions of Ser 514 and Gly 38 are according to Takahashi *et al.* (1986).

Cycle no.	Residues identified (pmol)	
	19 kDa fragment (from Ser 514)	51 kDa fragment (from Gly 38)
1	Ser 38	Gly 55
2	Arg —	Val 31
3	Ser 30	Asn 20
4	Asn 112	Leu 19
5	Val 115	Gln 15
6	Asp 102	Glu 11
7	Met 79	Phe 12
8	Asp 87	Leu 14
9	Phe 75	Asn 11
10	Glu 102	Val 6

Phe-Gln-Glu-Gly-Gln-Glu-Glu-Glu-Arg-Leu-Ala-Leu-Glu-Thr-Ala-Leu-Met-Tyr-Gly-Ala-Lys

Fig. 6. Amino acid sequence of a peptide purified from V8-proteinase digest of the 51 kDa fragment

This sequence is identical with amino acids 483–503 sequenced from placental Factor XIII (Takahashi *et al.*, 1986).

51 kDa fragment demonstrated that there was complete homology to amino acid residues Phe⁴⁸³–Lys⁵⁰³ in placental Factor XIII (Fig. 6). This peptide is therefore located in the C-terminal portion of the 51 kDa fragment.

DISCUSSION

Several investigators have reported that the a-chains of blood coagulation Factor XIII specifically bind to fibrin(ogen) (Lorand & Dickerman, 1955; Folk & Chung, 1975; Greenberg & Shuman, 1982; Skrzynia *et al.*, 1982; Greenberg *et al.*, 1985, 1987; Chung & Lewis, 1986). Since the a-chains from platelet, plasma and placental Factor XIII have similar amino acid compositions (Schwartz *et al.*, 1971; Ichinose *et al.*, 1986; Takahashi *et al.*, 1986), we used purified platelet Factor XIII to isolate a fibrin-binding fragment. Limited proteolysis by thrombin and trypsin demonstrated that the a-chain had three domains sensitive to proteolytic cleavage. Proteolysis at the N-terminus released a 4 kDa activation peptide and cleavage at the C-terminus released a 19 kDa peptide, leaving a central 51 kDa segment. These results are in agreement with the data on placental Factor XIII (Takahashi *et al.*, 1986). On the basis of amino acid sequence homologies of the N-terminal peptides of the 51 kDa and 19 kDa fragments with placental Factor XIII and the patterns of trypsin and thrombin cleavages, we have localized a fibrin-binding site to amino acid residues Gly³⁸–Lys⁵¹³ in the a-chain of Factor XIII.

Takahashi *et al.* (1986) and Schwartz *et al.* (1971) degraded Factor XIII by thrombin in the absence of Ca²⁺ and reported formation of a 56 kDa fragment that did not have transglutaminase activity. We found that the proteolysis of Factor XIII in the presence of Ca²⁺ preserves the transglutaminase activity of this fragment (Mary *et al.*, 1988). Attempts to renature the inactive 51 kDa fragment with Ca²⁺ have been unsuccessful (results not shown). Ca²⁺ ions apparently play a crucial role in maintaining the activity of the enzyme after the 19 kDa peptide is cleaved from the C-terminus. We have taken advantage of this property of Ca²⁺ to isolate a fibrin-binding fragment capable of cross-linking fibrin(ogen).

The three domains in the a-chains of platelet Factor XIII varied in their sensitivity to radioiodination: the central 51 kDa domain was radiolabelled, whereas the 4 kDa and 19 kDa peptides derived from the N- and C-termini respectively were not (Fig. 1). The absence of tyrosine residues from the 4 kDa activation peptide (Takagi & Doolittle, 1974; Takahashi *et al.*, 1986) explains its inability to be radioiodinated. However, we have been unsuccessful in our attempts to radiolabel the 19 kDa peptide even after trypsin digestion (C. S. Greenberg, A. Mary & K. E. Achyuthan, unpublished work). The tyrosine residues in the 19 kDa peptide (Ichinose *et al.*, 1986; Takahashi *et al.*, 1986) are probably inaccessible for iodination.

Limited trypsin proteolysis of platelet Factor XIII yielded peptide fragments that could be purified by gel-filtration h.p.l.c. under non-denaturing conditions. Following trypsin removal of the 19 kDa peptide from the C-terminus, the dimeric Factor XIII a-chains dissociated (Fig. 4). These data suggest that the presence of the 19 kDa peptide promotes the non-covalent association of the a-chains.

A 51 kDa fragment from Factor XIII was purified by gel-filtration h.p.l.c. This fragment, like intact Factor XIII, specifically bound to fibrin, expressed transglutaminase activity, cross-linked the α - and γ -chains of fibrin(ogen), retained the active-site cysteine residue and was radiolabelled. Both intact Factor XIIIa and the purified 51 kDa fragment could bind to fibrin even after the active-centre cysteine residue was covalently modified by iodoacetamide. Finally, the lysine analogue 6-amino-hexanoic acid, though competitively inhibiting plasminogen binding to fibrin (Wiman & Wallen, 1977), had no effect on the binding to fibrin of either Factor XIIIa or the 51 kDa fragment. This suggests that lysine residues are not involved in Factor XIIIa/51 kDa fragment binding to fibrin.

Identification of a 51 kDa fragment derived from the 80 kDa a-chain that retains fibrin-binding ability and transglutaminase activity has important implications for understanding the molecular basis of fibrin cross-linking. It has been debated whether both a-chains are required for transglutaminase function (Chung *et al.*, 1974; Curtis *et al.*, 1974). Because a monomeric 51 kDa fragment from the a-chain binds to fibrin and catalyses γ -glutamyl- ϵ -lysyl cross-links, each activated a-chain could independently cross-link fibrin. Alternatively, since Factor XIIIa contains a pair of catalytic and fibrin-binding sites, a single dimeric Factor XIIIa molecule could span two fibrin molecules and simultaneously catalyse two reciprocal cross-links. Additional studies are needed to define the molecular basis of fibrin cross-linking. The 51 kDa domain in Factor XIII could also function to localize Factor XIIIa to the fibrin clot and to regulate fibrin cross-linking.

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