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LOCALIZATION OF A SITE INTERACTING WITH HUMAN PLATELET RECEPTOR ON CARBOXY-TERMINAL SEGMENT OF HUMAN FIBRINOGEN γ CHAIN

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We report that the 27-residue carboxy-terminal cyanogen bromide fragment of human fibrinogen γ chain inhibits binding of [^{125}I]fibrinogen to human platelet receptors and blocks fibrinogen-mediated aggregation of ADP-treated human platelets. The blocking activity of the peptide was preserved after proteolysis of the isolated peptide with staphylococcal protease to generate a mixture of a dodecapeptide and a pentadecapeptide. Trypsin treatment destroyed blocking activity of the isolated peptide. These results indicate that the site responsible for the interaction of human fibrinogen with the platelet receptor resides in the 27-residue carboxy-terminal region of the γ chain.

Introduction

Fibrinogen, a clottable plasma glycoprotein participates in the cell adhesion phenomema involving prokaryotic and eukaryotic cells (1,2). It is essential for the interaction of platelets with surfaces and with each other, a phenomenon known as platelet aggregation (3,4). The covalent structure of human fibrinogen (Mr 340,000) comprising three pairs of polypeptide chains (α, β, γ) arranged in three general domains (central E and two terminal D) has been described (5). Localization of the binding region of human fibrinogen for the platelet receptor remained unknown until our recent demonstration that the γ chain (Mr 47,000) of human fibrinogen bears the main site for interaction with the platelet receptor (2). We follow this finding with data showing that the 27-residue carboxy-terminal cyanogen bromide fragment of γ chain inhibits the interaction of human fibrinogen with platelet receptors.

Materials and Methods

Human fibrinogen (Kabi, Sweden) was used without any further purification and [^{125}I]fibrinogen used for binding studies was prepared as before (2).

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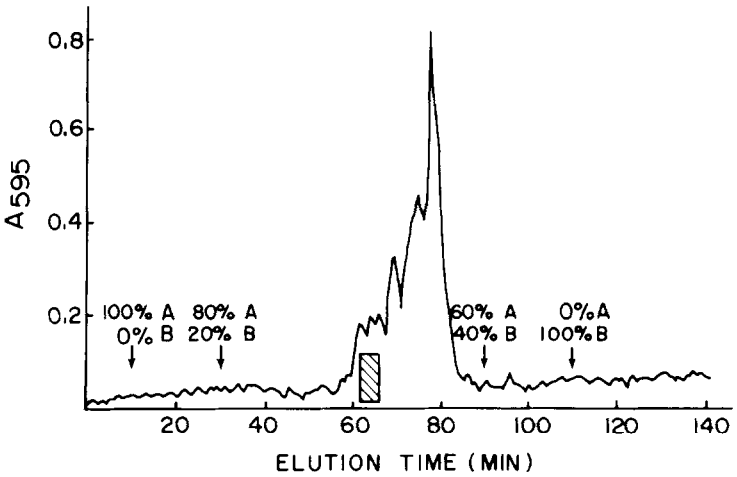

Fig. 1 HPLC preparative run of the water soluble peptides possessing inhibitory activity. The flow rate was 1.6mL/min. Solvent system used was: A - 0.1% trifluoroacetic acid, B - 0.1% trifluoroacetic acid in 80% acetonitrile. Arrows indicate time position when the linear gradient of A and B solvents or isocratic conditions were changed. Shaded area indicates fractions containing both inhibitory activities (See text).

Reduced and carboxymethylated chains (6) were dialyzed against 0.025M sodium acetate buffer pH5.2 with 8M urea and applied on a CM-Sepharose CL-4B (Pharmacia, Sweden) column (2 x 30cm) equilibrated with the same solvent. Chains were eluted using a linear gradient of sodium chloride, 0-0.3M, 1600mL, in 0.025M sodium acetate buffer, pH5.2, with 8M urea. Pooled chains were dialyzed against water and freeze-dried.

Purified γ chain preparation was degraded with cyanogen bromide (CNBr) in 70% formic acid for 6 hours, then freezed-dried and separated on a Sephadex G-50 column (2 x 100cm) equilibrated with 1% formic acid. Pooled fractions were treated with pyridine and water soluble peptides were extracted as described by Strong et al. (7).

Water soluble peptides demonstrating biological activity were subjected to high performance liquid chromatography (HPLC) on Whatman Partisil 10 ODS-3 Magnum 9 (0.9 x 50cm) and on Waters Associates μBondpak C18 (0.4 x 30cm) columns. The protein concentration was determined using Bradford reagent (8) with fibrinogen as a standard protein. Fractions containing peptide material were checked for their inhibitory activity in the staphylococcal clumping assay (1) and in the binding assay of [^{125}I]fibrinogen to human platelets treated with ADP as described previously (9).

Amino acid composition was determined on LKB automated amino acid analyzer. The peptide samples were hydrolyzed at 105°C for 22 hours with hydrochloric acid/propionic acid mixture (50:50 v/v) (Pierce Chemical Company, Rockford, Illinois). N-terminal sequencing of isolated peptides was carried out manually with 4-NN-dimethyloaminoazobenzene 4'-isothiocyanate (Pierce Chemical Company, Rockford, Illinois) as described by Chang et al. (10). In the case of biologically active peptide the N-terminal sequence was determined in Beckman automated sequencer as described by Misono and Inagami (11). Platelet-rich plasma was prepared from human citrated blood drawn freshly from healthy volunteers who were fasting and had abstained from taking aspirin or other medication during the preceeding 10 days. Platelets were immediately separated from plasma proteins by stepwise albumin gradient centrifugation and Sepharose 2B gel filtration as described (12). Platelets, suspended in Hepes buffer, pH7.35/0.1% dextrose/0.35% albumin, were adjusted to a final count of 1 x 10^8/0.5mL. Platelet aggregation was measured photometrically in a Payton dual channel aggregometer (Payton Associates, Buffalo, NY) as described by Born (13).

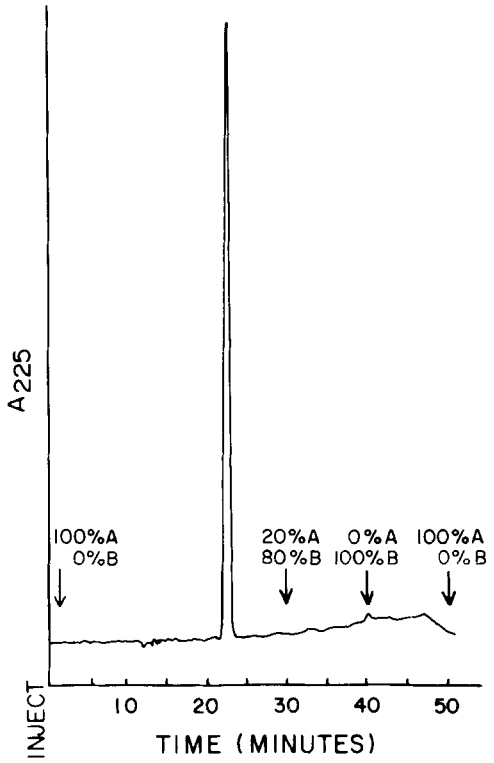

Fig. 2 HPLC analytical separation using 50nmoles of the peptide from the pool which is shaded in Fig. 1, with a flow rate of 1.0mL/min, and a solvent system as in Fig. 1.

Results

The procedure used previously (7) for isolation of the peptide inhibiting the staphylococcal clumping reaction with human fibrinogen was modified by using high pressure liquid chromatography to achieve a higher yield of isolated CNBr peptides derived from the γ chain of human fibrinogen. Gel filtration of γ CNBr peptides on a Sephadex G-50 column in 1% formic acid gave peptide pools similar to those described previously (7). Only one of these pools of water soluble peptides possessed inhibitory activity in the binding assay of [^{125}I]fibrinogen to human platelets treated with ADP and in staphylococcal clumping reaction. This pool was subjected to HPLC. Blocking activities toward both test systems were demonstrated in the same pool of peptides obtained from a HPLC preparative column (Fig. 1). Further separation of this pool on an analytical HPLC column (Fig. 2) produced only one sharp peak.

Amino acid composition of the purified peptide showed very good agreement with that of the 27-residue fragment located on the COOH-terminal of γ chain

TABLE I. Amino Acid Composition of γ-chain CNBr Peptide With Blocking Activity

Amino acid	CNBr-peptide moles/10^5g	CNBr-peptide moles/mole (nearest integer)	γ385-411[a] moles/mole
Aspartic acid[b]	75.3	2	2 (1)
Threonine	38.7	1	1
Serine	11.4	0	0
Glutamic acid[b]	153.9	4	4 (3)
Proline	-	0	1
Glycine	164.2	4	5
Alanine	72.3	2	2
Cystine	-	0	0
Valine	36.8	1	1
Methionine	-	0	0
Isoleucine	89.3	2	3
Leucine	74.7	2	2
Tyrosine	1.6	0	0
Phenylalanine	37.0	1	1
Histidine	62.6	2	2
Lysine	95.5	2	2
Arginine	21.7	1	1
Total	964.4	24	27

[a] data from the sequence of γ chain (5)
[b] aspartic acid and glutamic acid values include asparagine and glutamine (values in parenthesis) respectively.

(Table I). Data expressed in moles of amino acids per mole of peptide were obtained from molecular weight calculated from the amino acid sequence of the COOH-terminal 27 amino acid fragment (2500 daltons). Amino-terminal analysis (manual method) of active fractions from the preparative column (shaded area in Fig 1) showed the presence of a NH_2-terminal lysine residue followed by two isoleucine residues. This pattern corresponds to Lys-384, Ile-385, Ile-386 of the γ chain sequence (5). The peptide obtained from the analytical column (Fig. 2) was sequenced in an automated sequencer and showed that the first 10 amino acids were Lys-Ile-Ile-Pro-Phe-Asn-Arg-Leu-Thr-Ile in agreement with the sequence reported by Strong, et al. (7).

The inhibitory effect of the peptide on the binding of [^{125}I]fibrinogen to platelets was concentration dependent (Fig. 3). The inhibition curve reached saturation at 30 μM and the 50% inhibition of binding occured at approximately 7 μM peptide concentration. The peptide (0.4 mM) inhibited completely the aggregation of platelets induced by ADP and fibrinogen (Fig. 4).

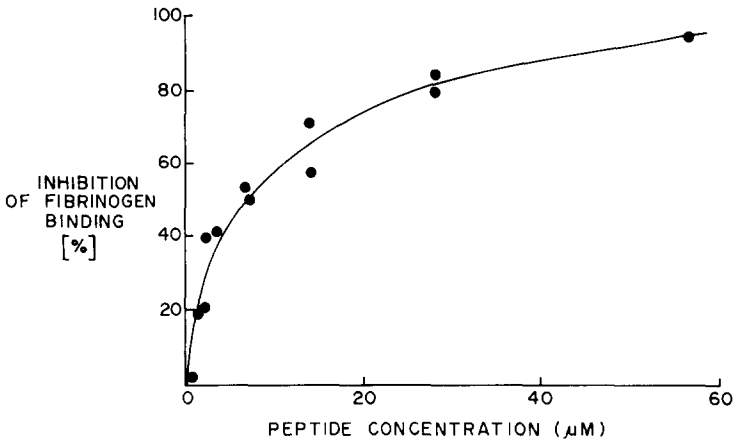

Fig. 3 Concentration dependent inhibitory effect of the 27-residue fragment on binding of [^{125}I]fibrinogen (0.16μM) to human platelets (1 x 10^8). Platelets were separated from plasma proteins and treated with ADP (5μM). Inhibition (%) was calculated after subtracting the value for binding of [^{125}I]fibrinogen to platelets without ADP.

Trypsin digestion (cf. 7) abolished the inhibitory activity of the isolated peptide tested in the [^{125}I]fibrinogen-platelet binding assay. On the other hand digestion with staphylococcal protease for 16 hours did not change the inhibitory activity of the peptide thus suggesting that the resulting pentadecapeptide competes with intact fibrinogen for the platelet receptor.

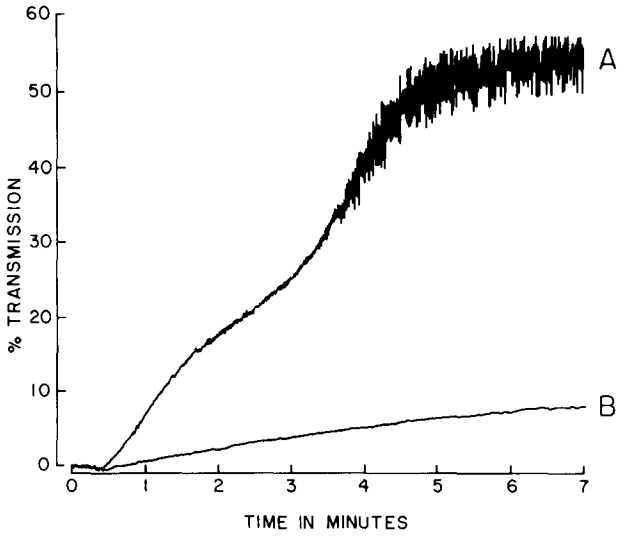

Fig. 4. Inhibition of platelet aggregation by the 27-residue fragment of γ chain. Human fibrinogen (0.16μM) and ADP (5μM) were added to induce platelet aggregation without the peptide (curve A) and with the peptide (curve B).

Discussion

We recently showed that among three distinct polypeptide chains (α, β, γ) of human fibrinogen the γ chain bears the main site reactive with the human platelet receptor (2). The approach developed in the previous study on identification of the site interacting with staphylococcal clumping factor was followed (7) and the material derived from the γ chain was analyzed in parallel for its reactivity toward the platelet receptor as well as toward the staphylococcal clumping receptor.

Experimental data presented in this paper show that the simple and fast two-step purification procedure: gel filtration of CNBr peptides on Sephadex G-50 and preparative HPLC of water-soluble pool of peptides (Fig. 1), gives a fraction of pure peptide possessing high blocking activity in human platelet aggregation induced by intact fibrinogen in the presence of ADP, and in the staphylococcal clumping reaction.

The purity of the isolated peptide is very high; it produces a single homogeneous peak on the analytical HPLC reverse phase column and it shows only one amino-terminal residue. The possible contamination detected in automated amino-terminal sequencing had to be a very small one, probably with some short hydrophilic peptide(s). Amino acid analysis and NH_2-terminal sequence data firmly establish that this peptide, reactive with human platelet receptor, encompassed the last 27 amino acid residues of the carboxy-terminal segment of the γ chain. This segment of the γ chain possesses several important features which are related to the overall function of the fibrinogen molecule. It encompasses cross-linking donor and acceptor sites (5) and it contains at least three bonds susceptible to serine proteases such as plasmin and trypsin (5). It has a site recognizing the staphylococcal clumping factor (7).

The concentration of the isolated carboxy-terminal peptide used to inhibit binding of [^{125}I]fibrinogen (0.16 μM) to platelets was achieved at approximately 7 μM of the peptide. This value represents 44 fold molar excess in regard to [^{125}I]fibrinogen in the binding system. In the platelet aggregation system 2500 fold molar excess of the peptide was needed. Digestion of this

peptide with trypsin abolishes completely its inhibitory activity, whereas digestion with staphylococcal protease does not. It means that this biological activity of fibrinogen requires continuity of the COOH-terminal pentadecapeptide segment of the γ chain composed of highly hydrophilic amino acids. This fragment of the γ chain does not contain any predominant type of secondary structure (Kloczewiak, unpublished results). Probably it represents an irregular type of structure fitting to receptors for fibrinogen on the platelet membrane as described in this report.

Acknowledgements

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