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A synthetic peptide with anti-platelet activity derived from a CDR of an anti-GPIIb-IIIa antibody

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Abstract A monoclonal antibody, AC7, directed against the RGD (Arg-Gly-Asp) binding site on the GpIIIa subunit of the platelet fibrinogen receptor, interacts only with activated platelet. In order to identify the regions of AC7 that interact with the receptor, cDNA sequences of AC7 immunoglobulin heavy and light chain variable regions were determined. Among the six complementarity-determining regions (CDRs) of AC7, the CDR3 heavy chain (H3) contains homology to the RGDF sequence within fibrinogen. A synthetic peptide encompassing the H3 region (H3, RQMIRGYFDV) inhibited platelet aggregation and fibrinogen binding to platelet (IC_{50} = 700 μM). The inhibitory potencies of modified H3 peptides suggest that the RGYF sequence within the H3 peptide mimic the receptor recognition sequence in fibrinogen.

Key words: Anti GpIIb-IIIa antibody; Immunoglobulin sequence; Synthetic peptide; Platelet aggregation inhibitor

1. Introduction

Binding of fibrinogen to its platelet receptor, the GpIIb-IIIa complex, depends on platelet activation by agonists such as ADP, thrombin or collagen; a process required for platelet aggregation. Fibrinogen binding to this glycoprotein is mediated in part by Arg-Gly-Asp-like sequences [1]. The RGD binding domain of GP IIb-IIIa has been localized in a fragment of the GPIIIa subunit that includes the sequence between amino acids 109–171 [2].

A murine monoclonal antibody, AC7 (IgM), has been produced against a synthetic peptide derived from the GPIIIa subunit (residues 109–128) and was shown to interact with platelets treated by a stimulus such as ADP. AC7/'activated' platelets interaction was inhibited by fibrinogen and RGD containing peptides.

Moreover, AC7 has been described to inhibit fibrinogen binding to its receptor and platelet aggregation in a dose-dependent fashion [3]. In order to characterize the structural features of AC7 responsible for its ability to inhibit platelet GpIIb-IIIa functions, we sequenced the heavy and light chain variable regions of AC7 immunoglobulin cDNAs, derived from AC7 mRNAs using a reverse-transcription polymerase chain reaction (RT-PCR) procedure. We assumed that amino acid sequences within the complementarity-determining-regions (CDRs) of the AC7 antibody could account for AC7 activity.

In this report we show that the CDR3 heavy chain (H3) of AC7 contains an RGYF motif which possesses structural and functional homology with the RGDF fibrinogen derived-peptide.

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Abbreviations: GpIIb-IIIa, glycoprotein IIb-IIIa; cDNA, complementary deoxyribonucleic acid; mRNA, messenger ribonucleic acid; SDS, sodium dodecyl sulfate.

2. Materials and methods

2.1. Polymerase chain reaction

Cytoplasmic RNAs were purified from the AC7 hybridoma cell line [4]. Immunoglobulin mRNAs were specifically reverse-transcribed using 3′ primers located on murine immunoglobulin heavy chain CH1 gene (amino acid residues 558–587, 5′-GGGAGACAGCAAGACCTGCGAGGTGGCTAG-3′, CH1) or on murine immunoglobulin light chain CK gene (residues 459–488, 5′-ACTGTTCAGGACGCCATTTTGTCGTTCACT-3′, CK). Primer sequences were designed to maximize homologies with published sequences [5] of μ heavy and K light chains of mouse immunoglobulin groups. Heavy chain variable region amplification by polymerase chain reaction (PCR) was performed using a 3′ primer (residues 414–443, 5′-GAAGTCCCGGGCCAGGCAGCCCATGGCCAC-3′, VH1) and a 5′ primer (residues 2–23, 5′-GGCTGCAGAGGTC/GA/CAA/GCTG/TCAGC/GAGTCA/TGG-3′, VH2 containing a *Pst*I restriction site –underlined). Light chain variable region was amplified using the 3′ primer CK1 and a 5′ primer (residues 1–24, 5′- CCGGATCCGACATTCAGCTGACCCAGTCTCCA-3′, VK1 containing a *Bam*HI restriction site – underlined). PCR products were then used for a second amplification procedure. A 3′ internal VH primer (residues 312–345, 5′-GGATCGATTGAGGAGACGGTGACCGTGGT-3′, VH3 containing a *Cla*I restriction site – underlined) and a 5′ primer (residues 1–23, 5′-GGCTGCAGCAGGTGCAGCTGAAGCAGTCAGG-3′, VH4 containing a *Pst*I restriction site – underlined) were used for amplification of the heavy chain variable region. The second 'light chain variable region amplification' was performed using a 3′ internal primer (residues 303–324, 5′-TCGAATTCGTTAGATCTCCAGCTTGGTCCC-3′, VK2 containing an *Eco*RI restriction site – underlined) and the 5′ primer VK1. All oligonucleotide primers were made on Pharmacia Cyclone synthetizer using phosphoramidite chemistry.

The second generated PCR products were digested with appropriate restriction enzymes, agarose gel-purified, subcloned into pBlue Script and sequenced on both strand by the chain termination method using the Sequenase kit (United States Biochemical Corp).

2.2. Microsequencing

After electrophoresis in SDS-polyacrylamide gel (10% SDS-PAGE) of purified AC7 immunoglobulin treated with 2% of β-mercaptoethanol, proteins were transferred onto an immobilon PVDF membrane (Millipore) according to the method of Matsudaira [6]. The N-terminal amino acid sequence of both K light and μ heavy chains were determined by automated Edman degradation methodology using an AB1 Mode 470A sequenator (Applied Biosystem).

2.3. Synthetic peptides

Synthetic peptides were obtained by solid-phase synthesis using an

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Applied Biosystems synthesizer. The peptides were more than 95% homogeneous when analyzed by high pressure liquid chromatography on a C18-ODS2 column eluted with a 0–80% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The amino acid composition of each peptide was consistent with its amino acid sequence.

2.4. Aggregation experiments

Platelets were isolated from fresh human blood drawn into acid/citrate/dextrose by differential centrifugation followed by gel filtration in a modified Tyrode buffer, pH 7.2, containing 2% bovine serum albumin (Calbiochem). Platelets (2×10^8/ml) in 360 μl of Tyrode buffer + 0.5 mM $CaCl_2$ were incubated, without stirring, in the presence of peptides. After incubation for 5 min, ADP (10 μM) and fibrinogen (0.1 μM) were added and aggregation was recorded at 37°C with a stirring rate of 1,000 rpm using a Payton dual-channel aggregometer (AHS, Strasbourg, France).

2.5. Binding experiments

Human fibrinogen was obtained by differential ether fractionation, as previously described [7]. Purified human fibrinogen was radiolabeled with carrier-free [^{125}I]Na (Amersham) using a modified chloramine-T procedure [8]. After removal of free iodine by filtration on P-20 column (Pharmacia) with phosphate buffer saline, the specific activity ranged from 0.5 to 2 μCi/μg of fibrinogen. The precipitability of radiolabeled fibrinogen in 10% thrichloroacetic acid was greater than 94%. Binding of radiolabeled fibrinogen to platelets was performed with 10^8 cells/ml

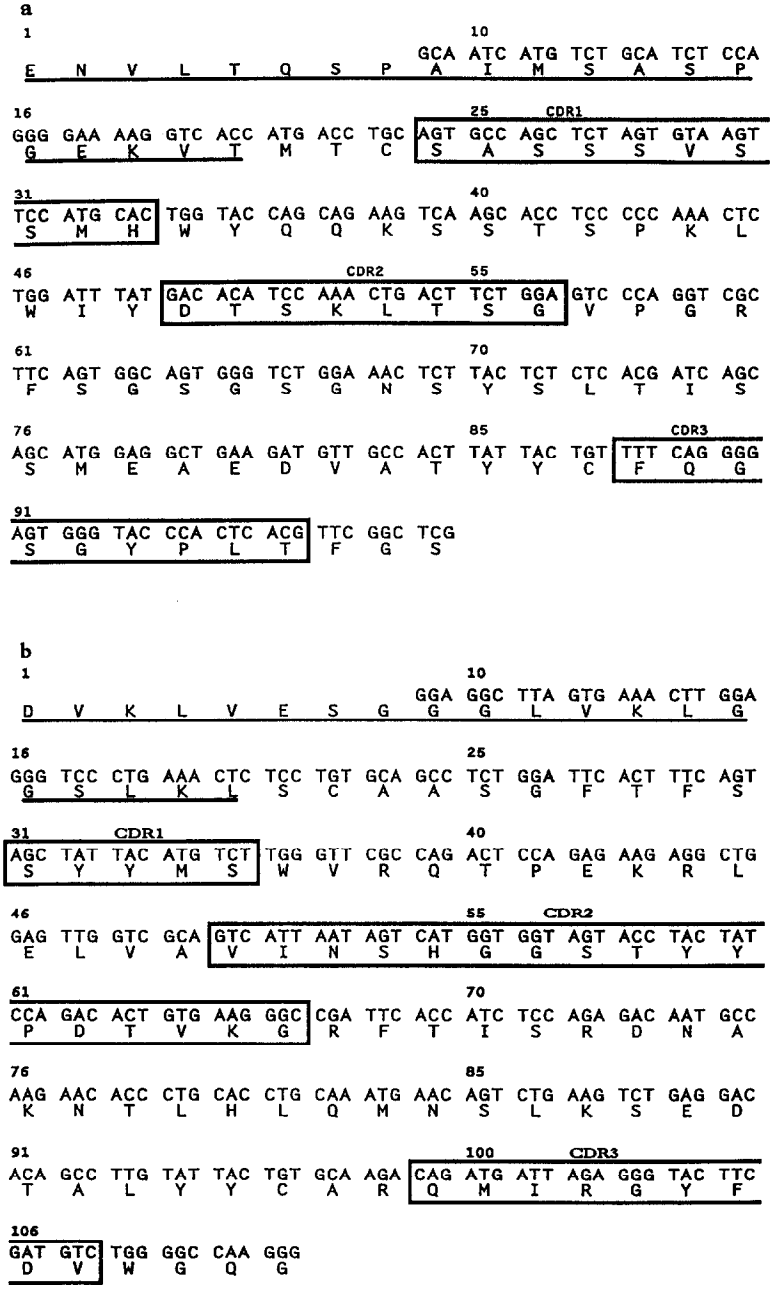

Fig. 1. Nucleotide and amino acid sequences of light and heavy variable regions of AC7 immunoglobulin. Nucleotide sequences of light (a) and heavy (b) variable regions of AC7 immunoglobulin were determined as described in section 2. The sequences were segregated into complementarity-determining regions (CDRs, boxes). Deduced amino acid sequences are shown using single letter amino acid code. N-terminal amino acid sequences determined by microsequencing are underlined (for details, see section 2). Comparison of amino acid sequences was performed using a 'Bisance' program.

in the presence of 0.5 mM calcium and 10 μM ADP. After 30 min of incubation, the bound ligand was separated from the free ligand by centrifugation of 80 μl samples through a 15% sucrose in Tyrode buffer and the radioactivity associated with platelets was quantified.

3. Results

3.1. The CDR3 of the AC7 heavy chain contains a motif similar to the fibrinogen A chain sequence

Light and heavy chain variable regions nucleotide sequences of AC7 immunoglobulin are listed along with the predicted amino acid sequences (Fig. 1). AC7 K light and μ heavy variable regions belong to class IV and III of mouse K and μ variable regions, respectively. Since, NS1 myeloma used for AC7 hybridoma production possess its own, but not secreted K light chain immunoglobulin, AC7 K light chain sequence was confirmed by N-terminal microsequencing (N-terminal microsequencing has been also monitored for the AC7 μ heavy variable region and confirmed nucleotide sequence data). The only similarity between AC7-K or AC7-μ sequences and fibrinogen was found in the CDR3-μ. This CDR3 sequence (RQMIRGYFDV) possesses analogy with the RGDF region of fibrinogen Aα chain, known as efficient inhibitor of AC7/platelet interaction [3]. This peptidic sequence was named H3 and further analyzed.

3.2. A synthetic peptide corresponding to H3 inhibits platelet aggregation and fibrinogen binding to platelets

In order to test the prediction that the QMIRGYFDV sequence within the AC7 heavy chain CDR3 plays a role in the recognition of AC7 immunoglobulin with the 'activated' fibrinogen receptor (GpIIb-IIIa), we synthetized a 10-residue peptide (H3: RQMIRGYFDV), and tested its ability to inhibit platelet aggregation (Fig. 2) and fibrinogen binding (Fig. 3) to ADP-stimulated platelets. H3 peptide inhibitory effect on platelet aggregation correlates with the inhibition of fibrinogen binding which results in a IC_{50} of 700 μM. The remaining CDR-derived peptides (H1, H2, K1, K2, K3) did not induced any inhibition of platelet activity. Inhibition of fibrinogen binding by RGDF was analyzed for comparison and the GRGESP peptide was used as a negative control [9].

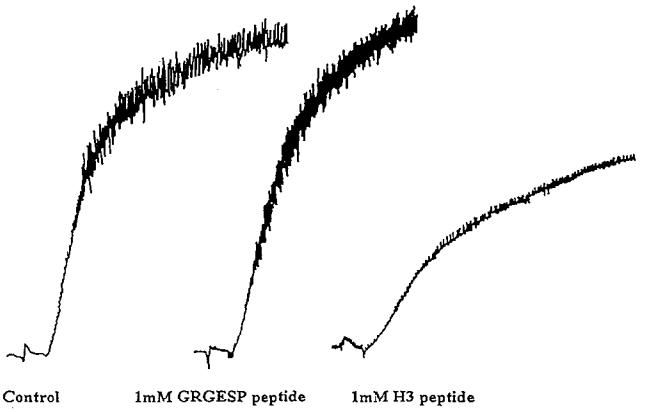

Fig. 2. Inhibition of platelet aggregation by H3 peptide. Aggregations were performed with platelets (2.10^8 cells/ml) in the presence of 0.5 mM $CaCl_2$. Platelets were incubated in the presence or in the absence (control) of 1 mM H3 (RQMIRGYFDV) or 1mM negative control peptide (GRGESP). After 5 min, aggregations were initiated by adding 10μM ADP, 0.1 μM fibrinogen and stirring.

3.3. Modifications of H3 peptide affect its inhibitory effect

Specific roles of sequences present into the H3 peptide are listed in Table 1. Deletion of the N-terminal portion of the H3 peptide (ΔH3 Y) reduced strongly its inhibitory effect on the binding of fibrinogen to ADP-stimulated platelets (ΔH3 Y, IC_{50} of 2,200 μM instead of 700 μM for H3 peptide). However, the central portion of the H3 peptide plays an important role: substitution of tyrosine (Y) to glycine (G) in the ΔH3 Y peptide produces an inactive coumpound (any inhibition is obtained even at 2 mM), while replacing tyrosine (Y) to aspartic acid (D) increased the inhibitory effect of the ΔH3 Y peptide (IC_{50} = 12 μM).

4. Discussion

The results presented in this report suggest that the RGYFDV sequence, within the CDR3 heavy chain variable region of AC7 IgM immunoglobulin mimics the RGD platelet receptor (GpIIbIIIa) recognition sequence in fibrinogen. However, this sequence was less potent than the antibody itself

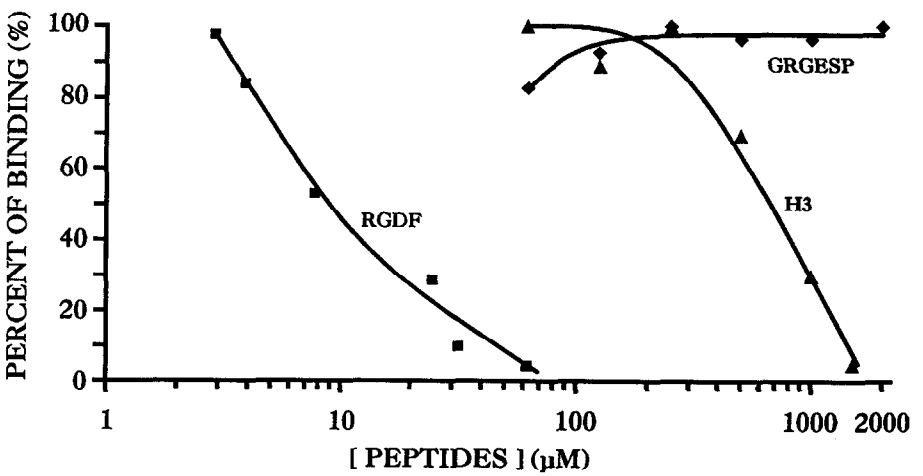

Fig. 3. Inhibition of [^{125}I]fibrinogen binding to ADP-stimulated platelets by H3 peptide. 10 μM ADP, 0.1 μM [^{125}I]fibrinogen and various concentrations of H3 (△), RGDF (□) or Fibrinogen binding was performed on platelets (10^8 cells/ml) in the presence of 0.5 mM $CaCl_2$, GRGESP peptides (♦). The GRGESP and RGDF peptides were used as negative and positive control, respectively.

Table 1
Inhibition of [^{125}I]fibrinogen binding to ADP-stimulated platelets by modified H3 peptides

Peptide	Inhibition of [^{125}I]fibrinogen binding (IC 50) μM
H3: RQMIRGYFDV	700
Δ H3 Y: RGYFDV	2,200
Δ H3 Y→G: RGGFDV	ND
Δ H3 Y→D: RGDFDV	12

Binding experiments were performed as described in Fig. 3.

(IC_{50} = 700 μM for the RQMIRGYFDV peptide versus IC_{50} = 105 nM for AC7 immunoglobulin for the binding of fibrinogen) [3].

An other IgM murine monoclonal antibody (PAC-1), that also inhibits fibrinogen binding to platelet [10] has been described to possesse an active RYD motif in its heavy chain CDR3 [11]. This sequence corresponds to the germline D gene DSP 2.10 and is assumed to determine the specificity of PAC-1 for fibrinogen receptor. Tomiyama and co-workers 12] studied extensively others antibodies that contains the same RYD-containing germline D gene DSP 2.10. As these antibodies show different affinities for GpIIb-IIIa, the authors concluded that the RGD-like sequence orientation and therefore accessibility into the different constrained CDR3 heavy chain variable region of the corresponding antibodies define their function. Moreover, NMR studies of constrained RGD containing peptides suggested that the RGD potency depends on conformation [3].

We report here the sequence of an analoguous anti GpIIb-IIIa antibody (AC7) which does not contain the RYD-containing DSP 2.10 sequence but exibits another RGD-like sequence (RQMIRGYFDV) in the corresponding CDR. It should be noted that the RQMIRGYFDV sequence within the whole AC7 immunoglobulin is folded as a constrained β-turn and interacts with other CDRs to form the binding recognition site of platelet fibrinogen receptor. Thus, knowledge of the tridimentional structure of AC7 heavy chain CDR3 within the entire immunoglobulin should lead to potent inhibitors of thrombosis.

These sequences are available from the EMBL data library under Accession Numbers X79553 (heavy chain) and X79554 (light chain).

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