Thrombospondin Binding to Specific Sequences within the Aα- and Bβ-Chains of Fibrinogen*

Thrombospondin is a multifunctional adhesive glycoprotein which binds to the surface of resting and activated platelets. Thrombospondin also binds to a variety of proteins, including fibrinogen. The interactions between platelet-bound thrombospondin and fibrinogen are thought to facilitate irreversible platelet aggregation. Both the Aα- and Bβ-chains of fibrinogen specifically bind to thrombospondin. Cyanogen bromide cleavage products of the fibrinogen Aα- and Bβ-chains, and synthetic peptides corresponding to specific regions of these cleavage products were utilized to identify the regions of the fibrinogen Aα- and Bβ-chains which bind to thrombospondin. Cyanogen bromide cleavage products of the Aα- and Bβ-fibrinogen chains, resolved by gel filtration and reversed-phase chromatography, were examined for thrombospondin binding activity. Thrombospondin specifically bound to the Aα-chain fragment encompassing residues 92-147 and the Bβ-chain fragment encompassing residues 243-305. Analyses of the binding characteristics of two series of overlapping synthetic peptides revealed that peptides corresponding to residues 113-126 of the Aα-chain and residues 243-252 of the Bβ-chain retained thrombospondin binding activity. Separate bovine serum albumin conjugates of the active Aα-chain and Bβ-chain peptides inhibited platelet aggregation. These studies reveal that fibrinogen possesses at least two unique sequences which are recognized by thrombospondin and that such interaction may affect platelet aggregation.

Platelet aggregation can be reversible or irreversible (1-3). Primary, or reversible, aggregation of stimulated platelets is mediated by the specific binding of fibrinogen to its receptor, active complexes of the platelet membrane glycoproteins Ib-IIIa (4-7). Primary aggregation occurs in the absence of secretion of the contents of platelet storage granules (1). Similarly, irreversible platelet aggregation is dependent upon fibrinogen binding to its receptors, but it is also secretion-dependent (1-3, 8). The mechanisms underlying irreversible platelet aggregation are not understood, but it is known that fibrinogen can become irreversibly bound to the platelet surface (9-11). The fact that irreversible aggregation is secretion-dependent, coupled with the observation that platelets from patients with the Gray platelet syndrome aggregate poorly in response to thrombin (12, 13), support the suggestion that proteins secreted from platelet α-granules help stabilize platelet aggregates and thereby facilitate irreversible platelet aggregation (3, 14, 15).

The receptor-mediated binding of thrombospondin to platelets is complex since both calcium-independent (16-19) and dependent (20) binding have been characterized. At least four thrombospondin receptors have been described including fibrinogen (21, 22), sulfated glycolipids (23), glycoprotein IV (24), and the vitronectin receptor (25, 26). The physiological roles, if any, of these receptors in platelet function have not been clarified. Although thrombospondin binds to fibrinogen, the binding of thrombospondin to platelets is not fibrinogen-dependent since thrombasthenic and normal platelets bind equivalent amounts of thrombospondin (19, 27).

A variety of evidence indicates that thrombospondin mediates, at least in part, the irreversible aggregation of platelets. Thrombospondin is the endogenous platelet lectin, a hemagglutinin found on the surface of platelets following secretion of their α-granule contents (8, 21, 28-33). Inhibitors of the hemagglutination activity of platelet-bound thrombospondin also inhibit platelet aggregation (28, 29). A variety of anti-thrombospondin polyclonal (34-36) and monoclonal (37-39) antibodies inhibit platelet aggregation and the fibrinogen-independent surface expression of thrombospondin (38). A polyclonal anti-glycoprotein IV antibody inhibits thrombospondin binding to platelets and platelet aggregation (40). Collectively, these studies have led to the proposal that thrombospondin may mediate irreversible platelet aggregation by binding to endogenous receptors on the platelets and to fibrinogen which is bound to glycoprotein IIIb-IIIa. Previous work by Bacon-Baguley et al. (41, 42) has shown that the Aα- and Bβ-chains of fibrinogen bind specifically to thrombospondin. The aim of this study is to identify the regions within these fibrinogen chains which contain the thrombospondin binding sites.

MATERIALS AND METHODS

Human fibrinogen was purchased from Helena Laboratories. Thrombospondin was isolated from A231W released bovine platelets as previously described (41-43). Thrombospondin was iodinated with Na125I (Amersham) in the presence of IODO-BEADS (Pierce) to a specific activity of 3.1 KBq/pmol thrombospondin chain, as described (41). Nickel electrophoresis and filter paper were obtained from Schleicher & Schuell; film for autoradiography was obtained from Eastman Kodak. Cyanogen bromide was purchased from Sigma. Chromatography solvents were procured from American Burdick and Jackson.

Separation of Fibrinogen Chains—Human fibrinogen was reduced and carboxymethylated (44, 45), dialyzed, and freeze-dried, and the individual chains resolved by reversed-phase chromatography on a Vydac C4 column (214TPS10). The column was equilibrated in 0.1% phosphoric acid: the elution buffer contained 0.1% phosphoric acid.

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in 50% acetonitrile. Approximately 10 mg of fibrinogen chains were separated per chromatography (Fig. 1). Fractions from each peak were pooled, dialyzed against 5% acetic acid, and freeze-dried.

Blot Binding Assay—The binding of iodinated thrombospondin to the fibrinogen chains was determined as previously described (41, 42). Fibrinogen fragments and peptides were assayed for $^{125}$I-thrombospondin binding employing a dot-blot binding assay using a 96-well Minifold microsample filtration manifold (Schleicher & Schuell). A 0.45-μm nitrocellulose sheet was moistened with distilled water, air dried, and placed in the manifold as part of a sandwich. The sandwich in the manifold was in order (top to bottom): buffer reservoir, filter support, filter paper, nitrocellulose, and 96-well sample plate. Freeze-dried samples were dissolved in TBS at a concentration of 0.25 μg/μl and 50–100 μl applied to a well in the manifold. Once applied to the wells, the samples were incubated for 15 min at room temperature prior to gentle negative pressure aspiration of the remaining sample into the well. The nitrocellulose sheet was removed from the manifold, placed in a solution of 4% bovine serum albumin in TBS, and incubated for 2 h. The nitrocellulose sheet was rinsed with 100 ml of TBS and then overlayed with $^{125}$I-thrombospondin (10⁶ cpm/ml) diluted in TBS. Following a 2-h incubation with $^{125}$I-thrombospondin, the nitrocellulose sheet was placed in a closed plastic container and manually washed with 5 × 200 ml of TBS, air-dried, secured to a filter paper, and placed in a x-ray cassette along with film.

Isolation of CNBr-cleaved Fibrinogen Chain Peptides—Purified fibrinogen Aα- and Bβ-chains were separately dissolved in 70% formic acid at a concentration of 5 mg/ml and a 200-fold (w/w) excess of CNBr was added; 18 h later, the samples were diluted and freeze-dried. CNBr-generated peptides were resolved by HPLC utilizing a combination of gel filtration and reversed-phase chromatography. Samples (1–2 mg) were applied to a Superose-12 column (Pharmacia LKB Biotechnology Inc.) equilibrated in 70% formic acid and resolved at a flow rate of 0.2 ml/min; elution was monitored at 280 nm. Fractions were lyophilized and assayed for thrombospondin binding employing the dot-blot binding assay. Fractions which bound thrombospondin were applied to a C18 column (PepRPC, HR5/5; Pharmacia) equilibrated in 0.1% trifluoroacetic acid; the column was eluted with a linear gradient to a final concentration of 90% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Fractions were freeze-dried prior to assay for thrombospondin binding.

Analytical Procedures—Amino acid analyses were obtained using Waters Associates Pico-Tag methodology employing precolumn phenylisothiocyanate derivatization (46, 47). Samples were vapor-phase-hydrolyzed for 75 min at 150 °C. Amino acid microsequence analysis was obtained by automated Edman chemistry on an Applied Biosystems model 477A protein sequencer. The abbreviations used are: TBS, Tris-buffered saline (50 mM Tris, 200 mM NaCl, pH 7.4); BSA, bovine serum albumin; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.
Biosystems gas-phase Model 470 sequenator with on-line, automated phenylthiodyantoin identification (44, 45).

Synthetic Peptides—Synthetic peptides were made using a PSS-80 automated synthesizer (Applied Protein Technologies, Cambridge, MA). Resins for solid phase synthesis were purchased from Bachem or Peninsula Laboratories. All D-butoxycarbonyl amino acids were obtained from Bachem except N-D-butoxycarbonyl-N'-mecitylene-2-sulfonyl)-L-arginine which was purchased from Applied Biosystems. Peptides were cleaved from the resin by trifluoromethane sulfonic acid (Sigma) (48). Following cleavage, peptides were filtered twice on a Sephadex G-15 column in 1 M acetic acid. Peptides which bound thrombospondin in the dot-blot binding assay and one corresponding control peptide were further purified by HPLC and subsequently tested for thrombospondin binding. Authenticity of each peptide was verified by amino acid analysis and sequence.

Platelet Aggregation—Platelet aggregation was performed in either platelet-rich plasma or with washed platelets (34, 46) using ADP or thrombin as the agonist, respectively. Peptides were added prior to agonist and evaluated for their ability to inhibit aggregation.

BSA-conjugated Peptides—BSA-conjugated peptides were also tested for their ability to inhibit platelet aggregation. These were prepared by incubating 5 mg of peptide and 5 mg of BSA with 35 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) at room temperature for 1 h followed by dialysis against distilled water (49). Relative concentrations of peptide/BSA conjugates were determined by the Bio-Rad protein assay (50). Since the β1 peptide produced extensive precipitation in the presence of BSA and carbodiimide, a modified β1 peptide was synthesized (NTENGGWTVIQNRQD) and conjugated to BSA. This modified peptide was more soluble than β1 and did not precipitate as much in the presence of BSA and carbodiimide.

RESULTS

Thrombospondin Binding Domains of Fibrinogen—The peptides generated from CNBr cleavage of the αA-chain of fibrinogen were fractionated by gel filtration (Fig. 3); fractions 6–8 (Fig. 2, inset) had the highest specific binding activity for 125I-thrombospondin. Amino-terminal sequence analysis of the pool of these fractions revealed at least 4 distinct residues. The thrombospondin binding peptides (fractions 6–8) from the gel filtration column were pooled and applied to a C18 reversed-phase column (Fig. 3); fractions 15–18 (Fig. 3, inset) retained the ability to bind thrombospondin. An amino-terminal sequence analysis of the pool of these fractions yielded a single series of phenylthiohydantoin residues which corresponded to the 56-residue CNBr peptide, residues 92–147 in the αA-chain (51) (Fig. 4).

The Bβ-chain of fibrinogen was fractionated by gel filtration and C18 reversed-phase chromatography following CNBr cleavage in a manner identical with that employed for the αA-chain. Fraction 11 from the gel filtration procedure (Fig. 5) bound 125I-thrombospondin (Fig. 5, inset). Amino acid sequence analysis of this fraction indicated that there were four separate peptides present in this fraction. This material was further resolved by C18 chromatography (Fig. 6) into two fractions (Fig. 6, inset) which accounted for all the recovered thrombospondin binding activity. Amino-terminal sequence analysis of these fractions yielded a single series of phenylthiohydantoin residues which corresponded to positions 242–305 of the Bβ-chain (51) (Fig. 7).

Synthetic Peptides of Fibrinogen—A set of overlapping peptides corresponding to the active 56-residue CNBr fragment of the αA-chain was synthesized. Each peptide of this set was tested for its ability to bind 125I-thrombospondin (Fig. 8). The peptide α-3, which contains residues 113–126 of the αA-chain of human fibrinogen (51), demonstrated the greatest binding. Similarly, overlapping peptides of the active 63-residue CNBr fragment of the Bβ-chain were synthesized and examined for thrombospondin binding (Fig. 9). The β1 synthetic peptide corresponding to residues 243–252 of the Bβ-chain of human fibrinogen accounted for all of the observed thrombospondin binding. The peptide representing residues 280–294 was made and tested separately due to technical difficulties encountered with its synthesis; it did not bind thrombospondin (data not shown).

Platelet Aggregation—Neither of the peptides (α-3 or β-1)
which bound thrombospondin tested separately or together (data not shown), inhibited platelet aggregation. Consequently, each peptide was conjugated to BSA and tested for its ability to inhibit thrombin- and ADP-induced aggregation. BSA-conjugated α-3 (EDLRSRIEVLKRKV) and modified β-1 (NTENGKWTVIQNRQD) inhibited thrombin-induced aggregation of washed platelets (data not shown). In platelet-rich plasma, the α-3 and β-1 conjugates primarily inhibited the secondary wave of ADP-stimulated aggregation (Fig. 10). The modified β-1 conjugate was a weaker inhibitor of aggre-
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Fig. 8. Binding of thrombospondin to synthetic Aα-peptides. The 56-residue sequence of the CNBr fragment from the Aα-chain was synthesized as a series of overlapping peptides (identified as peptides #1–#5) and tested for thrombospondin binding activity. Peptide 3, residues 113–126 of the fibrinogen Aα-chain, retained thrombospondin binding properties. Its position within this CNBr fragment is depicted in the upper portion. The autoradiogram, where each dot received 25 μg of fibrinogen (Fgn) or peptides #1 through #5 is shown in the center of the figure. The single-letter code for the peptide sequences is also presented.

$\text{FIG. 9. Binding of thrombospondin to synthetic Bβ-peptides.}$ The 63-residue sequence of the CNBr fragment from the Bβ-chain was synthesized as a series of overlapping peptides (identified as peptides #1–#6) and tested for thrombospondin binding activity. Peptide #1, residues 243–252 of the fibrinogen Bβ-chain, retained thrombospondin binding properties. Its position within this CNBr fragment is depicted in the upper portion. The autoradiogram, where each dot received 25 μg of fibrinogen (Fgn) or peptides #1 through #5 is shown in the center of the figure. The single-letter code for the peptide sequences is also shown. Peptide #5, corresponding to residues 280–294, was synthesized and tested separately; it did not bind thrombospondin.

**Fig. 10. Thrombin-induced platelet aggregation in the presence of thrombospondin-binding peptide conjugates.** Peptide/BSA conjugates were added to platelet-rich plasma (300 μl) and Ca²⁺-free Tyrode solution to a final volume of 500 μl prior to the addition of ADP (8 μM final). Aggregation was recorded for 3 min after the addition of ADP. a, 100 μg of α-4/BSA (no inhibition); b, no addition control; c, 200 μg of β-6/BSA (no inhibition); d, 200 μg of β-1*/BSA (35% inhibition); e, 100 μg of α-3/BSA (43% inhibition). β-1* = NTENGGWTVIQNRQD.
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Excellent technical assistance and Drs. Shinji Satoh and Rasheeda Zafar has made a similar observation. Others have reported inhibition of platelet aggregation by a monoclonal antibody which binds near the carboxyl terminus of thrombospondin (37). It is not understood how any of these antibodies inhibit platelet aggregation. Under certain conditions, purified thrombospondin promotes the aggregation of both nonstimulated and thrombin-stimulated platelets (38).

Our data not only support the concept that thrombospondin-fibrinogen interaction plays a role in platelet aggregation, but it also provides important information about the details of this interaction. The fact that two distinct peptides, each derived from the CNBr fragments of the Aα- and Bβ-chains of fibrinogen, bind to thrombospondin demonstrates that fibrinogen contains at least two independent, sequence-specific thrombospondin binding sites. The first site identified is contained within residues 113-126 of the Aα-chain of fibrinogen. The second site identified is found within residues 243-252 of the Bβ-chain. The binding of these sites on the Aα- and Bβ-chains of fibrinogen to thrombospondin may account for the ability of thrombospondin to stabilize platelet aggregates and thereby help convert reversible platelet aggregation into irreversible aggregation.

The ability of the ω3 peptide-BSA conjugate to inhibit platelet aggregation may result from a competition between the peptide components of the BSA conjugates and the fibrinogen Aα-chain for thrombospondin. According to this view, the BSA-peptide conjugate binds to thrombospondin and thereby prevents the Aα-chain of fibrinogen from binding. However, the ω3 peptide was not an effective inhibitor of platelet aggregation unless derivatized to BSA.

Both the ω3 and ω1 regions of fibrinogen may be required for binding to thrombospondin during platelet aggregation. Following ω3/BSA binding to thrombospondin on the platelet surface, the affinity of fibrinogen for thrombospondin may be greatly reduced if the ω1 region is the only site on fibrinogen which can now participate in thrombospondin binding. Alternatively, inhibition by ω3/BSA may be due both to competition with fibrinogen Aα-chains and additionally to steric hindrance of Bβ-chain binding to thrombospondin by the relatively large BSA molecule. A similar argument can be made for the ω1/BSA conjugates.

The results presented here demonstrate that the thrombospondin binding activity of fibrinogen may be explicable in terms of two independent binding sites, each composed of a contiguous sequence of amino acids. Our data do not exclude the possibility that CNBr treatment of fibrinogen chains destroyed other thrombospondin binding sites, nor does it exclude the possibility that binding sites exist in intact fibrinogen which do not exist in the single chains. Further study is required to evaluate these possibilities. Nonetheless, the simplest interpretation is that all the binding activity of fibrinogen may reside in the two binding sites described in this study.

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