Affinity of Fibrinogen Binding to Platelet Membrane Glycoprotein IIb/IIIa Increases with RGDS and γ Chain Fibrinogen Peptide Hybrid

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Abstract. Arg-Gly-Asp (RGD)-containing peptides and the peptide unique to fibrinogen in the C-terminal domain of the γ chain are important for fibrinogen binding to platelet membrane glycoprotein (GP) IIb/IIIa. We synthesized a unique hybrid peptide of YRGDSPLGGAKQAGDV encompassing the RGD sequence (RGDS) with the γ chain peptide (LGGAKQAGDV). Maximum binding to thrombin-stimulated platelets was achieved within 40 minutes with the YRGDS peptide and within 20 minutes with the hybrid peptide and native fibrinogen. The platelet binding sites were 58,600 molecules with the hybrid peptide and 52,400 molecules with YRGDS. These peptides inhibited fibrinogen binding to thrombin-stimulated platelets in a dose-dependent manner. The order of inhibitory potency of these peptides was as follows: fibrinogen - hybrid peptide > YRGDS > GQQHHLGGAKQAGDV (G15). The RGES peptide had no inhibitory activity. These three peptides inhibited binding of the anti-GP IIb/IIIa monoclonal antibody (LJ-CP8) to platelets. The hybrid peptide showed the most potent inhibitory activity, with an IC50 of 48 μM. These results suggest that the linear combined RGDS and γ chain peptide created with proline increases the affinity of binding to activated platelets.

Key Words. fibrinogen, GP IIb/IIIa, affinity, synthetic peptides

Platelet membrane glycoprotein (GP)IIb/IIIa forms a calcium-dependent heterodimer complex that serves as an activation-dependent receptor for adhesive proteins, including fibrinogen [1-3], fibronectin [4-6], von Willebrand factor (vWF) [7,8], and vitronectin [9-11]. The interactions of these adhesive proteins with platelet receptors mediate or modulate the platelet attachment, spreading, and aggregation reactions. Binding of fibrinogen to GPIIb/IIIa mediates platelet aggregation, serving a crucial role in thrombus formation [8].

Proposed binding sites to GPIIb/IIIa on the fibrinogen molecule are a decapeptide (Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val; LGGAKQAGDV) at the C-terminal domain of the γ chain [12-14] and two Arg-Gly-Asp (RGD) sequences in the α chain (RGDS sequence at 572-575 and RGDF at 95-98) [15-17]. The RGD sequence has been demonstrated to be crosslinked predominantly to GPIIIa [18-22]. The binding domain of the γ chain is located in GPIIb [1,19,23]. These observations suggest that binding of fibrinogen to GPIIb/IIIa occurs through RGD-GP IIIa and the γ chain segment-GPIIb.

The affinity of these peptides for platelets is less than that of native fibrinogen. Modified peptides that increase the affinity for GPIIb/IIIa are good tools for understanding the interaction of the receptor recognition sites of fibrinogen and GPIIb/IIIa. In this study we have synthesized a hybrid peptide encompassing sequences from α- and γ-chains, and have evaluated the affinity of hybrid peptide to thrombin-stimulated platelets to investigate the relationship of these receptor recognition domains of fibrinogen to GPIIb/IIIa.

Materials and Methods

Human plasma fibrinogen was purchased from Sigma Chemical (St. Louis, MO). Na125I was purchased from Amersham (Arlington Heights, IL). Anti-GP IIb/IIIa monoclonal antibody, designated LJ-CP8, which inhibits binding of fibrinogen to GP IIb/IIIa [24], was a kind gift from Dr. Zaverio M. Ruggeri from the Scripps Institute (La Jolla, CA). Immunoglobulin G was purified from ascitic fluid by affinity chromatography on protein A-Sepharose CL-6B (Sigma, St. Louis, MO) [25].

Synthesis and purification of synthetic peptides

The hybrid peptide of YRGDSPLGGAKQAGDV was synthesized on a peptide synthesizer at TANA Bio-Systems LC (Houston, TX). Propidol was purchased from Amersham (Arlington Heights, IL). Anti-GP IIb/IIIa monoclonal antibody, designated LJ-CP8, which inhibits binding of fibrinogen to GP IIb/IIIa [24], was a kind gift from Dr. Zaverio M. Ruggeri from the Scripps Institute (La Jolla, CA). Immunoglobulin G was purified from ascitic fluid by affinity chromatography on protein A-Sepharose CL-6B (Sigma, St. Louis, MO) [25].

Synthesis and purification of synthetic peptides

The hybrid peptide of YRGDSPLGGAKQAGDV was synthesized on a peptide synthesizer at TANA Bio-Systems LC (Houston, TX). It should be noted that proline was inserted between RGDS and LGGAKQAGDV. YRGDS, RGES, and the peptide of

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GQQHHLGGAKQAGDV at the C-terminus of the γ chain were purchased from Bachem Fine Chemicals (Torrance, CA). Lyophilized crude peptides were purified by reverse-phase HPLC on a C18 column, using an elution gradient of 0–80% acetonitrile with 0.1% trifluoroacetic acid. The purity and composition of the peptides were verified by HPLC analysis of hydrolysates prepared by treating the peptides under nitrogen with 6 N HCl containing 0.1% phenol at 160°C. The sequences shown use a single-letter amino acid code as follows: K = lysine, R = arginine, H = histidine, E = glutamic acid, D = aspartic acid, Q = glutamine, N = asparagine, P = proline, G = glycine, S = serine, T = threonine, V = valine, I = isoleucine, L = leucine, Y = tyrosine, A = alanine, F = phenylalanine, C = cysteine, W = tryptophan, M = methionine.

**Preparation of washed platelets and radiolabeling**

Blood was drawn into a solution of acid-citrate-dextrose (ACD) (0.318 M citric acid, 0.062 M trisodium citrate, 0.133 M glucose) anticoagulant and centrifuged to obtain platelet-rich plasma (PRP). The platelets were washed using the albumin density gradient technique, as described elsewhere [26], with modification [24]. Washed platelets were resuspended in modified Tyrode’s buffer (5 mM HEPES, 0.15 M NaCl, 2.5 mM KCl, 12 mM NaHCO₃, 5.5 mM glucose) containing 0.2% bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO).

Human fibrinogen was labeled with carrier-free ¹²⁵I using Iodo-Gen (Pierce Chemical, Rockford, IL) [27]. Unbound reagent was removed by gel filtration on a 0.5 x 20 cm column of Sepharose G-25 equilibrated with TBS (20 mM Tris-HCl, 0.15 M NaCl, pH 7.3). The peptides were also radiiodinated using the same method as for fibrinogen. The iodinated peptides were separated from free Na¹²⁵I and other reagents by gel filtration on a 10 ml G-10 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in Tris-buffered saline (TBS), as described elsewhere [28].

**Binding studies**

Binding of ¹²⁵I-fibrinogen (30 µg/ml) or labeled peptides to thrombin-stimulated washed platelets was performed as previously described [24]. In brief, washed platelets (1 x 10⁸/ml) were activated by 0.1 U/ml thrombin in the presence of 1.0 mM CaCл₂. Following incubation for 10 minutes at 22°C, thrombin activity was blocked with a 30-fold excess of hirudin (Sigma Chemical, St. Louis, MO). Various concentrations of ligand and labeled materials were then added. The mixture was incubated for 30 minutes at 22°C without agitation. The interaction was quantitated by separating bound from free ligand by centrifugation of 50 µl of the mixture through 300 µl of 20% sucrose at 12,000 x g for 4 minutes. Binding was expressed as a percentage of that measured in a control mixture in the absence of inhibitor, after subtraction of non-specific binding.

Nonspecific binding was evaluated in each experiment by adding a 50-fold excess of unlabeled ligand. The concentration of a competing substance required to inhibit specific binding by 50% (IC₅₀) was then calculated from dose-response curves in which the percentage of residual binding was plotted against the logarithm of competing ligand concentration.

**Protein concentration**

Concentrations of proteins were measured by the method of Bradford using BSA as the standard [29]. The actual peptide concentration was measured by the bicinchonic acid assay (Pierce Chemical) with BSA as the standard.

**Results**

**Effect of synthetic peptides on ¹²⁵I-fibrinogen binding to thrombin-stimulated platelets.**

All peptides were soluble in TBS. YRGDS and the γ chain peptide inhibited fibrinogen binding to thrombin-stimulated platelets in a dose-dependent manner, with IC₅₀ of 17 ± 1.6 µM and 104 ± 6.8 µM, respectively. RGDS peptide was approximately five times more potent than the γ chain peptide.

The hybrid peptide of YRGDSPLGGAKQAGDV used in this study also inhibited fibrinogen binding to stimulated platelets with an IC₅₀ of 0.81 ± 0.16 µM, similar to that of native fibrinogen with IC₅₀ of 0.38 ± 0.08 µM. The control peptide RGES had no inhibitory

![Fig. 1. Effect of synthetic peptides on ¹²⁵I-fibrinogen binding to thrombin-stimulated platelets. ¹²⁵I-fibrinogen at a concentration of 30 µg/ml was added with varying concentrations of fibrinogen or peptides to washed platelets (1 x 10⁸/ml) in Tyrode’s buffer with 1.0 mM CaCl₂. The platelets were stimulated with 0.1 U/ml thrombin. Thrombin activity was blocked with a 30-fold excess of hirudin. The binding was measured after incubation for 30 minutes at 22°C and was expressed as the percentage of that measured in a control mixture containing Tris buffer instead of peptide. The results represent the mean of three determinants.](image-url)