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The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets

(fibronectin/cell attachment/synthetic peptide/receptor)

EDWARD F. PLOW*, MICHAEL D. PIERSCHBACHER†, ERKKI RUOSLAHTI†, GERARD A. MARGUERIE‡, AND MARK H. GINSBERG*

*Division of Inflammation and Vascular Biology, Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037; †Cancer Research Center, La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, CA 92037; and ‡Institut National de la Santé et de la Recherche Médicale U-143, Institut de Cellulaire Pathologie, Hopital Kremlin Bicetre, France

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ABSTRACT **The Arg-Gly-Asp sequence resides in the cell attachment region of fibronectin. Arg-Gly-Asp-containing peptides support fibroblast attachment, inhibit fibroblast adhesion to fibronectin, and inhibit fibronectin binding to thrombin-stimulated platelets. In view of the similarities between the binding of fibronectin, fibrinogen, and von Willebrand factor to stimulated platelets, we have examined the effects of Arg-Gly-Asp-containing peptides on the interaction of these latter two adhesive proteins with platelets. Gly-Arg-Gly-Asp-Ser-Pro was used as a prototype peptide, and this hexapeptide inhibited fibrinogen binding to ADP and thrombin-stimulated platelets in the 10–200 μM range. The inhibition exceeded 90% at high concentrations of peptide and was observed in the presence of either calcium or magnesium. Platelet aggregation was also inhibited by the peptide in this dose range. The hexapeptide inhibited fibrinogen binding to platelets with receptors fixed in an exposed state, indicating direct interference with the ligand–platelet interaction. The peptide was 1/2 to 1/3rd as potent in inhibiting fibrinogen as fibronectin binding to platelets, but fibrinogen and von Willebrand factor binding were inhibited to an identical extent. Conservative amino acid substitutions for the arginine, glycine, or aspartic acid markedly reduced inhibitory activity and the Asp-Gly-Arg sequence was inactive. These results indicate that Arg-Gly-Asp-containing peptides can inhibit the binding of the three adhesive proteins to stimulated platelets, establishing a basic common feature between the interaction of these molecules with platelets.**

Platelet attachment, spreading, and aggregation on extracellular matrices are central events in thrombus formation. These events can be regulated by a family of platelet adhesive glycoproteins—fibrinogen, fibronectin, and von Willebrand factor (vWF). Fibrinogen is a cofactor for platelet aggregation (1–3), fibronectin supports platelet attachment and spreading reactions (4–7), and vWF is important in platelet attachment to and spreading on subendothelial matrices (7–9). At a mechanistic level, the role of these adhesive proteins in platelet functions can be attributed to their interaction with specific binding sites on the cell surface. Although binding of these proteins to resting platelets is not detected, saturable interactions can be demonstrated with platelets stimulated by agonists such as thrombin (10–15). The binding of all three proteins to thrombin-stimulated platelets is divalent ion dependent (11, 14, 16, 17), and platelets from patients with Glanzmann thrombasthenia exhibit decreased capacity to bind all three proteins (11, 18, 19). Synthetic peptides corresponding in sequence to the carboxyl-terminal region of the γ chain of fibrinogen inhibit the binding of all three molecules to platelets (20), suggesting that common mechanisms may be involved in these interactions.

Recent studies have demonstrated that the Arg-Gly-Asp sequence within fibronectin is involved in the cell attachment function of this glycoprotein (21, 22). Arg-Gly-Asp-containing peptides support fibroblast attachment and inhibit attachment of these cells to fibronectin (21–23). We have recently shown that Arg-Gly-Asp-containing peptides inhibit the binding of fibronectin to thrombin-stimulated platelets, and the same structure–function relationships necessary for such peptides to support fibroblast attachment were required for inhibition of fibronectin binding to platelets (24). The Arg-Gly-Asp sequence exists in a number of other proteins (22, 23). Several of these proteins support cell attachment, and synthetic Arg-Gly-Asp-containing peptides have been shown to inhibit specific functions associated with these proteins (22, 25, 26). One protein containing the Arg-Gly-Asp sequence is fibrinogen, in which it resides at residues 95–97 and 572–574 of the 611-amino acid Aα chain (27, 28). The presence of an Arg-Gly-Asp sequence within vWF has also been recently demonstrated (29). In view of this sequence homology and the common features of the interaction of platelet adhesive proteins with the cell, we have examined the effects of Arg-Gly-Asp-containing peptides on the binding of fibrinogen and vWF to platelets.

MATERIALS AND METHODS

Adhesive Proteins–Platelet Interactions. Human platelets were isolated from fresh blood collected into acid citrate/dextrose by differential centrifugation followed by gel filtration on Sepharose 2B in divalent ion-free Tyrode's buffer (pH 7.4) containing 2% bovine serum albumin as described (10, 14, 16). The adhesive proteins were isolated from fresh human plasma. Fibrinogen was isolated by differential ethanol precipitation and ammonium sulfate fractionation (30). In addition, the fibrinogen preparations were then passed over a gelatin-Sepharose column to remove fibronectin. Fibronectin was isolated by gelatin-Sepharose affinity chromatography (31). vWF was purified from the cryoprecipitates of fresh-frozen plasma by gel filtration on Sepharose 2B (32). Each of the adhesive proteins was radioiodinated as described (20). The radiolabeled ligands were bound by their respective monospecific antibodies but not by antibodies to the other two adhesive proteins. Platelet binding of each of these radiolabeled proteins as performed in this study has also been described (20). Briefly, platelets were suspended at 1×10^8 per ml in the modified Tyrode's buffer to which a selected divalent ion concentration was added. The platelets were stimulated with 10 μM ADP or α-thrombin at 0.1 unit/ml. With thrombin stimulation, the

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Abbreviation: vWF, von Willebrand factor.

5-fold excess of hirudin was added 5 min after the stimulus. The radiolabeled proteins were then added at final concentrations of 0.3 μM for ^{125}I-labeled fibrinogen and ^{125}I-labeled fibronectin, and 0.4 μg/ml for ^{125}I-labeled vWF. Platelet-bound ligand was separated from free ligand by centrifugation through 20% sucrose for 3 min in a Beckman microfuge. As noted (20), nonspecific binding, as measured by the residual binding of ^{125}I-labeled ligand in the presence of a 50-fold excess of nonlabeled ligand or as the binding to nonstimulated cells, was low and did not exceed 10% of the total binding. Therefore, the results presented have not been corrected for these low levels of nonspecific binding. The ID_{50} is the dose of a particular peptide inhibiting the binding of a radiolabeled adhesive protein by 50% relative to a control lacking the peptide. For selected experiments, platelets were stimulated for 5 min with 10 μM ADP or with 0.1 unit of thrombin per ml, and then treated with 2% paraformaldehyde to obtain ADP- or thrombin-fixed platelets. These fixed platelet preparations express fibrinogen binding sites in an exposed state such that the platelet agonists are no longer required for receptor function (24, 33, 34). During the course of these studies with ^{125}I-labeled fibrinogen added at its apparent K_d of 0.3×10^{-6} M, the fibrinogen molecules bound per platelet ranged from 14,400 to 22,500 for ADP-stimulated; 12,800 to 18,500 for ADP fixed; 18,900 to 27,500 for thrombin-stimulated; and 16,800 to 24,300 for thrombin fixed platelets. With ^{125}I-labeled vWF added at 400 ng/ml, 420–850 ng were bound per 10^9 thrombin-stimulated platelets.

Synthetic Peptides. Peptides were synthesized by Peninsula Laboratories (San Carlos, CA), Bachem Fine Chemicals (Torrence, CA), or on a Model 430A peptide synthesizer from Applied Biosystems (Foster City, CA). The purity of these peptides was assessed by high-performance liquid chromatography on a Waters apparatus using a C_{18} μBondapak column with acetonitrile gradients. In addition, 24-hr hydrolyzates of the peptides were subjected to amino acid analyses on an automated Beckman amino acid analyzer. Two preparations of the prototype peptide, Gly-Arg-Gly-Asp-Ser-Pro were used in this study, and each was >95% homogenous by these criteria.

Platelet Aggregation. Washed human platelets were suspended at a final concentration of 1×10^8 platelets per ml in Tyrode's buffer containing 1 mM calcium, 0.3 μM fibrinogen, and the selected dose of Gly-Arg-Gly-Asp-Ser-Pro. Aggregation was initiated by addition of 10 μM ADP and was measured in a dual-channel aggregometer (Sienco DP-247E, Morrison, CO) at 37°C at a stirring rate of 1000 rpm as described (10).

RESULTS

Gly-Arg-Gly-Asp-Ser-Pro has been used as a prototype of the Arg-Gly-Asp-containing peptides, and its effects on fibrinogen binding to platelets has been examined (Fig. 1). In this analysis, ^{125}I-labeled fibrinogen added at 0.3×10^{-6} M, its K_d (10), to thrombin or ADP-stimulated fixed or nonfixed platelets. At 30 min, a time at which ^{125}I-labeled fibrinogen binding is at apparent steady state, the peptide produced a dose-dependent inhibition of fibrinogen binding to all cell preparations. With nonfixed platelets, the extent of inhibition was very similar with either platelet agonist; 50% inhibition (ID_{50}) was obtained at peptide concentrations of 30–40 μM, and $\geq$90% inhibition was observed with 200 μM peptide. Dose-dependent inhibition of ^{125}I-labeled fibrinogen binding to both fixed platelet preparations was also observed. Such fixed platelet preparations express fibrinogen receptors in an exposed state (24, 33, 34), indicating that the peptide can directly inhibit fibrinogen binding to its exposed receptor. Since higher concentrations of the peptide were required (ID_{50} = 80–95 μM) for inhibition of ^{125}I-labeled fibrinogen binding to the fixed platelets, an additional effect of the peptide on platelet stimulation and/or induction of the fibrinogen binding sites may also occur.

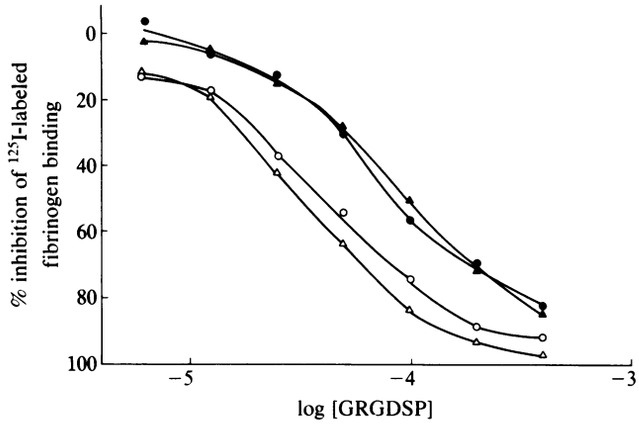

FIG. 1. The effect of Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) on fibrinogen binding to nonfixed and fixed platelets. Fixed platelets were prepared by stimulating the cells with 10 μM ADP (●) or with thrombin (0.1 unit/ml) (▲) for 5 min prior to treatment with 2% paraformaldehyde. These fixed platelets as well as fresh platelets stimulated with ADP (○) or thrombin (△) were used in the analysis at 1×10^8 platelets per ml. ^{125}I-labeled fibrinogen (0.3 μM) and various doses of GRGDSP were added to the stimulated platelets in Tyrode's buffer (pH 7.4) containing 2% albumin, 1 μM magnesium, and 1 μM calcium. Binding was measured after 30 min at 22°C, and percentage inhibition was calculated relative to fibrinogen binding to platelets in the absence of the hexapeptide. In the absence of peptide, the molecules of ^{125}I-labeled fibrinogen bound per platelet were as follows: ADP-stimulated, 18,300; thrombin-stimulated, 24,500; ADP-fixed, 16,400; and thrombin-fixed, 18,500.

As shown in Fig. 2, Gly-Arg-Gly-Asp-Ser-Pro inhibited the steady-state binding of ^{125}I-labeled fibrinogen to ADP-stimulated platelets in the presence of either calcium or magnesium. The peptide appeared to be slightly more inhibitory in the presence of magnesium/EGTA, but suppression was readily observed in the presence of either divalent ion.

Agents that inhibit fibrinogen binding to platelets should also interfere with platelet aggregation (12, 35). This prediction is documented with Gly-Arg-Gly-Asp-Ser-Pro (Fig. 3*A*). At a high dose of the peptide (400 μM), platelet aggregation induced by 10 μM ADP was completely inhibited, but the decrease in light transmission indicative of the change in platelet shape from a discoid to a more spherical form was not affected. In Fig. 3*B*, the effect of various doses of Gly-Pro-Gly-Asp-Ser-Pro on the initial rate of platelet aggregation and on the inhibition of ^{125}I-labeled fibrinogen binding to platelets has been correlated. In the range of 30 to 200 μM Gly-Arg-Gly-Asp-Ser-Pro, these events were linearly related.

The effect of Gly-Arg-Gly-Asp-Ser-Pro on vWF binding to platelets was examined. Various doses of the peptide were assessed for their capacity to inhibit fibrinogen, fibronectin, and vWF binding to the same preparation of thrombin-stimulated platelets (Fig. 4). The peptide inhibited vWF binding in a dose-dependent manner, and its capacity to inhibit fibrinogen and vWF binding was virtually identical. The peptide was, however, 3-fold more potent in inhibiting fibronectin binding (ID_{50} = 9 μM for fibronectin vs. 27 μM for fibrinogen and vWF). A similar relationship was also observed with thrombin-stimulated fixed platelets (not shown). At 100 μM Gly-Arg-Gly-Asp-Ser-Pro, vWF binding to platelets in the presence of ristocetin was not inhibited (Fig. 4).

The structural specificity of the inhibition of fibrinogen binding by Arg-Gly-Asp-containing peptides was analyzed and is summarized in Table 1. The tetrapeptides Arg-Gly-Asp-Ser (peptide 6) and Arg-Gly-Asp-Val (peptide 7) were

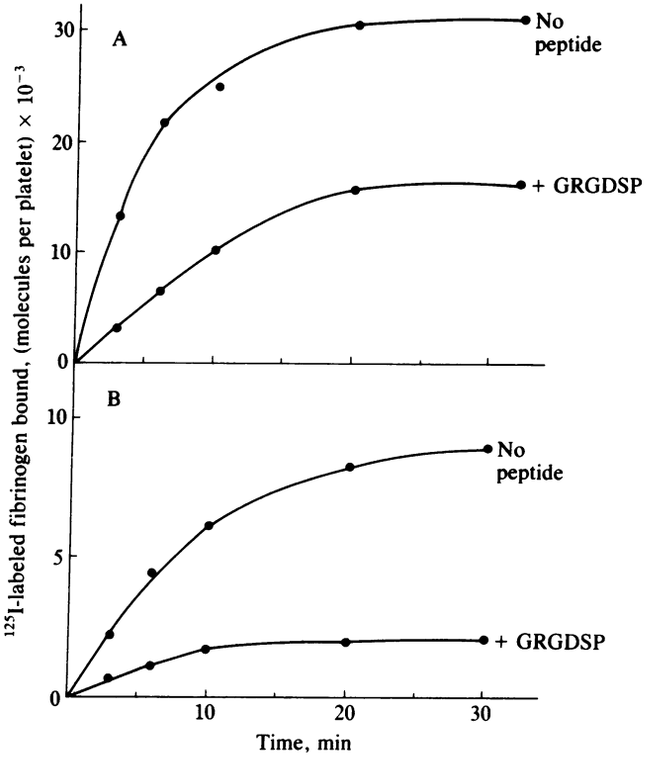

FIG. 2. The effect of Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) on the time course of fibrinogen binding to ADP-stimulated platelets. GRGDSP was 30 μM, ^{125}I-labeled fibrinogen was 0.3 μM, ADP was 10 μM, and platelets were at 1 × 10^8 per ml. (*A*) Modified Tyrode's buffer contained 1 mM calcium. (*B*) Modified Tyrode's buffer contained 2 mM magnesium and 5 mM EGTA.

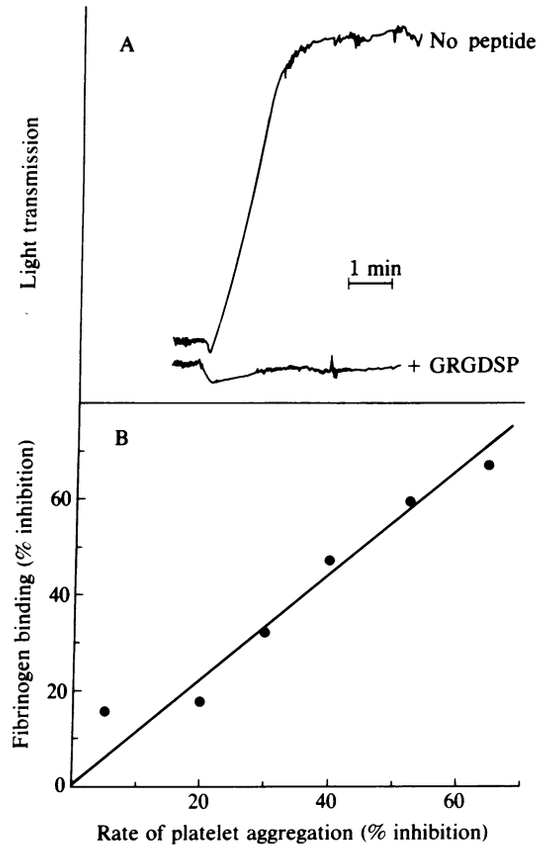

FIG. 3. The effect of Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) on platelet aggregation. (*A*) ADP-induced platelet aggregation was measured in the presence or absence of 400 μM GRGDSP. (*B*) Inhibition of the initial slope of platelet aggregation and of ^{125}I-labeled fibrinogen binding to ADP-stimulated platelets was measured in the presence of various doses of GRGDSP in the 20–100 μM range. In either case, percentage inhibition was calculated relative to the samples lacking the hexapeptide.

inhibitory, slightly more so than the hexapeptide (peptide 1), indicating the essential role of Arg-Gly-Asp sequence for the effect. The reversal of this sequence (peptide 8) resulted in an inactive peptide, indicating that it is the sequence rather than the individual amino acids that establishes inhibitory activity. Conservative substitutions for these amino acids—glutamic acid for aspartic acid in peptide 2, alanine for glycine in peptides 3 and 4, and lysine for arginine in peptide 5—decreased inhibitory activity by at least a factor of 10. Peptide 9 (Arg-Gly-Asp-Val) corresponds in sequence to the last four amino acids of the γ chain of fibrinogen, γ408–411. As previously reported (20, 36, 37), γ-chain peptides of 10–12 amino acids can inhibit the binding of fibrinogen, fibronectin, and vWF to stimulated platelets as do the Arg-Gly-Asp peptides. The only apparent primary structural homology between the two peptide sets is the Gly-Asp within peptide 9, and this peptide was noninhibitory.

DISCUSSION

In this study, we have examined the effects of Arg-Gly-Asp-containing peptides on fibrinogen binding to platelets. The prototype peptide, Gly-Arg-Gly-Asp-Ser-Pro, inhibited fibrinogen binding at doses ≥15 μM and produced >90% inhibition at 200 μM. Paralleling this effect, the peptide inhibited platelet aggregation in the same dose range. The key sequence for inhibition of the platelet–fibrinogen interaction was Arg-Gly-Asp. Since reversal of this sequence resulted in an inactive peptide, an effect of the amino acids *per se* on the interaction is excluded. Relatively conservative substitutions—lysine for arginine, alanine for glycine, and glutamic acid for aspartic acid—reduced or abolished inhibitory activity. These structure–function relationships are very similar to those we have previously noted for fibronectin binding to platelets (24) and for fibroblast attachment activity (22, 23). vWF and fibrinogen binding were inhibited to an identical extent by Gly-Arg-Gly-Asp-Ser-Pro, compatible with a similarity in their binding to stimulated platelets (20, 37, 38). These observations, coupled with our previously reported data on the inhibition of fibronectin binding by these peptides (24), provide direct evidence for a closely related binding mechanism for these adhesive proteins to platelets. It is noteworthy that the Arg-Gly-Asp peptides were 2- to 3-fold more potent in inhibiting fibronectin, as contrasted to fibrinogen and vWF binding to platelets. This result contrasts with the identical effect of the fibrinogen γ-chain peptides on platelet binding of the three adhesive proteins (20) and suggests that the mechanism of inhibition by Arg-Gly-Asp may not be precisely identical for each protein.

A primary effect of the Arg-Gly-Asp peptides on platelet stimulation and induction of adhesive protein receptors appears to be reasonably excluded by the following observations. (*i*) The platelets responded to ADP by undergoing a characteristic change in shape in the presence of high concentrations of the hexapeptide. (*ii*) Fibrinogen binding induced by two diverse stimuli, thrombin and ADP, was inhibited to the same extent. (*iii*) We have previously shown that these doses of Arg-Gly-Asp-containing peptides do not inhibit thrombin-induced secretion of serotonin from platelets (24). (*iv*) The peptide inhibited fibrinogen binding to platelets with exposed receptors. We have recently provided preliminary evidence to show that Arg-Gly-Asp-containing

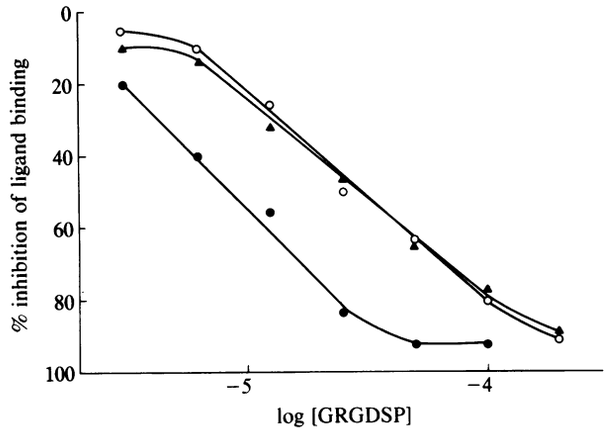

FIG. 4. The effects of various doses of Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) on fibrinogen (○), fibronectin (●), and vWF (▲) binding to thrombin-stimulated platelets. Washed platelets were stimulated with 0.1 unit of thrombin per ml in modified Tyrode's buffer containing 1 mM magnesium and 1 mM calcium. ^{125}I-labeled fibrinogen and ^{125}I-labeled fibronectin were present at 0.3 μM, and ^{125}I-labeled vWF was present at 400 ng/ml. Binding was measured after a 30-min incubation at 37°C. In the absence of peptide, the thrombin-stimulated platelets bound 27,500 ^{125}I-labeled fibrinogen and 48,000 ^{125}I-labeled fibronectin molecules per platelet, and 650 ng of ^{125}I-labeled vWF per 10^9 platelets. The single point (△) is the effect of the peptide at 100 μM on ^{125}I-labeled vWF binding in the presence of ristocetin at 0.1 mg/ml.

peptides bind directly to platelets (39). Specifically, we have shown that an Arg-Gly-Asp-containing heptapeptide bound to platelets with a K_d of 9×10^{-6} M, similar to its K_i for inhibition of fibronectin binding to platelets, and 58,000 binding sites per platelet were estimated (39). These observations are consistent with the inhibition of adhesive protein binding by these peptides being mediated by the interaction of the Arg-Gly-Asp-containing peptides with the cell. Similar preliminary evidence has been provided for binding of the fibrinogen γ-chain peptides to platelets (36). Thus, Arg-Gly-Asp peptides and fibrinogen γ-chain peptides may bind to platelets, exhibit a fine structural specificity for inhibitory activity, and are effective in inhibiting adhesive protein binding in the micromolar range. The issue as to whether these peptide sets define the same, related, or distinct recognition specificities is not presently known. It should be noted in this regard that the Ala-Gly-Asp-Val peptide, the only apparent primary structural homology between the two peptide sets, did not inhibit fibrinogen binding to platelets. The interrelationships between the binding sites defined by these peptide sets can best be resolved when the specific interactions of each with the platelet becomes more clearly defined and when the receptors for the adhesive proteins become isolated.

Table 1. Structural–functional relationships for the inhibition of fibrinogen binding to ADP-stimulated platelets by Arg-Gly-Asp-containing peptides

Peptide	ID_{50}, μM
1. GRGDSP	35
2. GRGESP	>1000
3. GRADSP	800
4. GRADSPC	420
5. GKGDS	450
6. RGDS	18
7. RGDV	15
8. CPSDGRG	>1000
9. AGDV	>1000

The single-letter amino acids are as follows: G, glycine; R, arginine; D, aspartic acid; S, serine; P, proline; E, glutamic acid; A, alanine; C, cysteine; K, lysine; and V, valine.

It has been suggested that Aα chains as well as γ chains of fibrinogen can interact with the platelet (40). It is unlikely that the Arg-Gly-Asp sequence at Aα 572–574 interacts with the platelets with high affinity. This conclusion is based on the data of Peerschke and Galanakis (41), which showed that fibrinogen degradation products lacking major segments of the carboxyl terminus of the Aα chain interact with platelets with a similar affinity as intact fibrinogen. Indeed, in preliminary experiments, we found that the Arg-Gly-Asp peptides inhibited the binding of such fibrinogen degradation products to platelets (unpublished results). The Arg-Gly-Asp sequence at Aα 95–97 remains a possible region for interaction with the cells. Less likely candidate sequences are Arg-Gly-Lys, Arg-Pro-Asp, and Arg-Gly-Gly of the Aα chain, Lys-Gly-Asp of the Bβ chain, and the two Ala-Gly-Asp sequences within the γ chain. The recent identification of an Arg-Gly-Asp sequence in vWF (29) further emphasizes the need to precisely evaluate the role of this peptide sequence in the platelet binding functions of the adhesive proteins. While the definitive role of these peptide sequences in these interactions remains to be elucidated, it can be unequivocally concluded from the present study that Arg-Gly-Asp-containing peptides inhibit fibrinogen, fibronectin, and vWF binding to platelets.

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