Fibronectin Inhibits Platelet Aggregation Independently of RGD Sequence

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Fibronectin binds to platelet membrane glycoprotein (GP) IIb-IIIa in Arg-Gly-Asp (RGD)-dependent and -independent manners. We have isolated and characterized the 29-kDa dispase fragment of fibronectin. Binding of $^{125}$I-fibronectin to thrombin-stimulated platelets was inhibited by the 29-kDa fragment and the GRGDSPA peptide with IC$_{50}$ values of 1.5 ± 0.4 and 8.1 ± 0.9 μM, respectively. The NH$_2$-terminal sequence of this fragment gave this result: Ala-Val-Thr-Thr-Ile-Pro-Ala-Pro-Thr-Asp. This established the position of this peptide within fibronectin as beginning with the residue tentatively designated 1597. Neither the RGDS sequence nor the RGD-independent binding domain of fibronectin (Bowditch, R. D., Halloran, C. E., Aota, S., Obara, M., Plow, E. F., Yamada, K. M., and Ginsberg, M. H. (1991) J. Biol. Chem. 266, 23323-23328) was contained in this fragment. The 29-kDa fragment inhibited ADP-induced aggregation of platelets and binding of fibrinogen to activated platelets. The fragment bound to immobilized GPIIb-IIIa. The $^{125}$I-labeled 29-kDa fragment directly bound to thrombin-stimulated platelets with 98,000 ± 4,600 molecules/platelet ($K_r = 4.6 ± 0.5 \times 10^{-9}$ M). Direct binding was inhibited by the unlabeled 29-kDa fragment but was not blocked by either the GRGDSPA peptide or the monoclonal anti-GPIIb-IIIa antibody. These results indicate that the additional RGD-independent binding domain(s) to GPIIb-IIIa are present on fibronectin. This additional binding domain(s) on fibronectin may prevent thrombus formation by interfering with the interaction of fibrinogen with GPIIb-IIIa.

Platelet membrane glycoprotein (GP) IIB-IIIa is a receptor for fibrinogen, which is thought to link the aggregating platelets together (1). Other adhesive proteins such as fibronectin may also contribute to platelet adhesion (2, 3) and aggregation (4). It has been shown that fibronectin binds to specific divalent cation-dependent receptors on thrombin-stimulated platelets (5, 6). Binding of fibronectin is inhibited by anti-GPIIb-IIIa monoclonal antibodies (7) and by short peptides (8, 9). These peptides contain the sequence Arg-Gly-Asp (RGD) from the cell-binding domain of fibronectin (10). Furthermore, fibronectin can cross-link to GPIIb-IIIa on platelets stimulated by thrombin or ADP (5). Finally, phospholipid vesicles that contain GPIIb-IIIa adhere to wells coated with fibronectin (11). GPIIb-IIIa functions as a potential platelet receptor for fibrinogen as well as fibrinogen (12). Based on these observations, the RGD sequence is thought to be the key attachment site and GPIIIa-IIIa a fibrinogen receptor.

Other findings suggest that fibrinogen and fibronectin bind to separate sites. 1) Whereas fibrinogen binds to ADP- or epinephrine-stimulated platelets (13), fibrinogen does not (6); 2) fibronectin does not inhibit binding of fibrinogen to platelets stimulated by ADP or thrombin, although fibrinogen inhibits binding of fibronectin to platelets (14). Different determinants within fibronectin are involved in the cell adhesion mediated by fibronectin. Recent study has demonstrated that the fragments, which spanned fibronectin residues 1359-1436, bound to GPIIb-IIIa in a divalent cation-dependent and RGD-independent manner (15). These observations indicate that multiple sites in fibronectin are involved in its recognition by GPIIb-IIIa.

Cells adherent on RGD-containing fragments and heparin-binding (RGD-independent) domains display fully developed focal adhesion (18). This suggests that the heparin-binding region of fibronectin may contain additional binding domain(s) to integrins. In this paper, we purified and characterized a monomeric 29-kDa dispase fragment of fibronectin using heparin affinity purification, beginning with Ala-1597. Although this fragment does not contain the RGD sequence and the RGD-independent binding domain (residues 1359-1436), it inhibits binding of fibrinogen to thrombin-stimulated platelets and binds to immobilized GPIIb-IIIa. Furthermore, this fragment inhibits platelet aggregation induced by ADP. These results suggest that fibronectin sequences in addition to RGDS and the sequence (residues 1359-1436) are involved in high affinity interaction between this ligand and GPIIb-IIIa.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human fibronectin and the peptides GRGDSPA and GRGESP were purchased from Bachen Chemical Co. Ltd. (Torrance, CA). Phenylmethylsulfonyl fluoride was purchased from Calbiochem, Na$_2$EDTA was purchased from Amersham Corp.

Monoclonal antibodies were the generous gifts of Dr. Zaverio M. Ruggeri (Scripps Institute, La Jolla, CA). Anti-GPIIb-IIIa monoclonal antibody (LJ-CP8) inhibits the binding of fibrinogen to GPIIb-IIIa (17). Anti-GPIIb monoclonal antibody (LJ-1b1) inhibits binding of von Willebrand factor to GPIIb (18). Anti-fibronectin polyclonal antibody (PK-2) has previously been described in detail (19). Immuno-globulin G was purified from ascitic fluid by affinity chromatography on protein A-Sepharose CL-4B (20).

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1 The abbreviations used are: GP, glycoprotein; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.
Amino-terminal amino acid sequence analysis of the 29-kDa fragment first lane. Total dispase digestive fragments of fibronectin, fragments with Coomassie Brilliant Blue. Purified fibronectin is shown in the first lane. Total dispase digestive fragments of fibronectin, segments are shown in lanes 2, 3, and 4, respectively.

**Table I**

*Table I* Amino-terminal amino acid sequence analysis of the 29-kDa fragment of fibronectin

| Cycle no. | Amino acid | Yield (pmol) |
|-----------|------------|-------------|
| 1         | Ala        | 29.7        |
| 2         | Val        | 21.9        |
| 3         | Thr        | 6.4         |
| 4         | Thr        | 7.8         |
| 5         | Ile        | 16.3        |
| 6         | Pro        | 16.7        |
| 7         | Ala        | 17.2        |
| 8         | Pro        | 14.3        |
| 9         | Thr        | 6.2         |
| 10        | Asp        | 14.0        |
| 11        | Leu        | 12.2        |

**Generation and Characterization of a Monomeric 29-kDa Dispose Digestive Fibronectin**—A 29-kDa dispase fragment of human fibronectin was purified by heparin affinity chromatography. In brief, fibronectin in 10 mM Tris, 0.15 M NaCl, 0.2 mM CaCl₂, and 0.02% (w/v) sodium azide, pH 7.4, was incubated with dispase (0.3 mg/mg fibronectin; neutral protease, grade I, Boehringer Mannheim, Germany) for 6 h at 22 °C. The reaction was terminated by the addition of 0.01 M EDTA. The digest was loaded onto a 1.5 × 5-cm column of heparin-Sepharose CL-6B equilibrated at 22 °C in TBS. After extensive washing with TBS, the 29-kDa fragment was eluted with 10 mM Tris, 0.5 M NaCl, and 0.02% (w/v) sodium azide, pH 7.4. The fragment was dialyzed exhaustively against TBS at 4 °C. The purity of intact fibronectin and of the 29-kDa fragment was verified by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

The amino-terminal end of the 29-kDa fragment was sequenced using an Applied Biosystems model 470A gas-phase sequenator. The resultant phenylthiohydantoin-derivatives were identified by isotropic high pressure liquid chromatography as previously described (21, 22).

**Preparation of Washed Platelets and Radiolabeling of Proteins**—Human platelets were obtained from fresh acid citrate dextrose-anticoagulated blood as described elsewhere (23). The platelets were washed three times in modified Tyrode’s buffer (5 mM HEPES, 0.15 M NaCl, 2.5 mM KCl, 12 mM NaHCO₃, 5.5 mM glucose).

Purified proteins were labeled with carrier-free ¹²⁵I (Amersham Corp.) using Iodo-Gen (Pierce Chemical Co.) (24). Unbound reagent was removed by gel filtration on a 0.5 × 20-cm column of Sepharose G-25 equilibrated with TBS.

**Binding Studies**—Bindings of fibronectin and fibrinogen to thrombin-stimulated washed platelets were performed as previously described (6, 23). In brief, washed platelets (1 × 10⁹/ml) were activated by 0.1 unit/ml thrombin in the presence of 1.0 mM CaCl₂. Following incubation for 10 min at 22 °C, thrombin activity was blocked with a 30-fold excess of hirudin (Sigma). Various concentrations of ligand and labeled fibronectin or fibrinogen were then added. The mixture was incubated for 30 min at 22 °C without agitation. The interaction was quantitated by separating bound from free ligand by centrifugation at 12,000 × g for 4 min. Binding was expressed as a percentage of that measured in a control mixture in the absence of inhibitor, after subtracting the nonspecific binding. Binding of fibrinogen to ADP-stimulated platelets was also evaluated with 5 μM ADP.

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.

**Aggregation Studies**—Platelet aggregation was carried out in siliconized cuvettes as described previously (26) using a platelet aggregation tracer (Nikkou Bioscience, Tokyo, Japan) at 37 °C and stirred at 1,000 rpm. Purified fibrinogen (0.5 mg/ml, 1.5 μM) and CaCl₂ (1.0 mM) were added to washed platelets (3 × 10⁶/ml) together with various concentrations of the 29-kDa dispase fragment of fibronectin.

**Table II**

| Inhibition of fibronectin binding to thrombin-stimulated platelets | Inhibition of fibrinogen binding to platelets^a^ (IC₅₀) |
|---------------------------------------------------------------|---------------------------------------------------|
| 29-kDa fragment (μM)                                          | 1.5 ± 0.4                                        |
| GRGDSPA (μM)                                                 | 8.1 ± 1.9                                        |
| GRGESP4A (μM)                                                | >500                                             |
| LJ-CP8 (μg/ml)                                               | 1.3 ± 0.3                                        |
| LJ-Ib1 (μg/ml)                                               | >200                                             |

^a Values are expressed as means ± S.D.
RGD-independent Fibronectin Binding to Platelets

Figure 3. Inhibition of ADP-induced platelet aggregation. Purified fibronectin, the 29-kDa fragment of fibronectin, synthetic peptides, or buffer were incubated with washed platelets (3 x 10^8/ml) in the presence of 1.0 mM CaCl_2. Aggregation was induced by adding 5 μM ADP (upper arrows). Aggregation was monitored as an increase in light transmission through the stirred platelet suspension.

ADP (5 x 10^-4 M) was then added. Aggregation was recorded as the increase in the light transmission through the mixture.

Studies on the 29-kDa Interaction with Insolubilized GPIIb-IIIa—Enzyme-linked immunosorbent assay was performed as previously described (27). Polystyrene microtiter wells were coated with either purified GPIb-IIIa or GPIb at a final concentration of 2 μg/ml. GPIb and GPIIb-IIIa were purified according to the methods described elsewhere (28, 29). To measure the interaction of the 29-kDa dispase fragment with these glycoproteins, the fragment was incubated at increased concentrations in the wells coated with the glycoproteins for 2 h at 22°C. Purified IgG of a selected anti-fibronectin polyclonal antibody (PK-2) was then added, and the plates were incubated an additional 60 min. The wells were washed three times with PBS, 0.05% Tween 20 (PBS-Tween), 100 μl of horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, Inc., South San Francisco, CA; 1:500 dilution in PBS-Tween) was added to each well, and the plates were incubated for 60 min at 22°C. The wells were washed three times, the peroxidase substrate (o-phenylenediamine; Zymed, Inc., South San Francisco, CA) was added, and absorbance at 482 nm was recorded.

Protein Concentration—Protein concentrations were measured by Bradford's (30) method using bovine serum albumin as a standard. The actual peptide concentration was measured using the bicinchoninic acid assay (Pierce chemical Co.) with bovine serum albumin as a standard.

RESULTS

Characterization of a 29-kDa Fibronectin Fragment—Treatment of fibronectin with dispase yielded a number of discrete fibronectin fragments. A single fragment with apparent molecular mass of 29,000 daltons under both nonreducing and reducing conditions could be purified by gel filtration following heparin affinity chromatography (Fig. 1). Approximately 2 mg of the fragment could be isolated reproducibly from 20 mg of purified fibronectin.

About 50 pmol of the 29-kDa fragment was immobilized to polyvinylidene difluoride membrane and sequenced directly on gas-phase sequenator. A single phenylthiohydantoin-derivative was detected at each cycle (Table I). An amino-terminal sequence was obtained for the 29-kDa fragment. The first 10 amino acids were as follows: Ala-Val-Thr-Thr-Ile-Pro-Ala-Pro-Thr-Asp. This sequence was compared with a tentative amino acid sequence of the molecule (31, 32) and was found to correspond in its entirety to the sequence beginning with alanine 1597 and ending with aspartic acid 1606. This sequence was not found elsewhere in the molecule. The RGDS sequence is not present within this fragment, and there is no apparent homology between the RGDS sequence and the 29-kDa fragment.

Inhibition Binding—Fibronectin is well known to bind to thrombin-stimulated platelets (5, 6, 33). Binding of fibronectin to thrombin-stimulated platelets was dose dependently

Figure 4. Direct binding of the 29-kDa fragment of fibronectin to thrombin-stimulated platelets. Upper panel, binding of the 29-kDa fragment to thrombin-stimulated platelets. Washed platelets (1 x 10^8/ml) were stimulated with thrombin at a concentration of 0.1 unit/ml. Nonspecific binding was determined by adding a 30-fold excess of cold 29-kDa fragment. Binding was measured after incubation for 30 min at 22°C. Data points represent the mean ± S.D. of three determinations. Lower panel, Scatchard plot of the data.
inhibited by the 29-kDa dispase fragment of fibronectin, the GRGDSPA peptide, and the monoclonal anti-GPIIb-IIIa antibody (LJ-CP8), with IC_{50} values of 1.5 ± 0.4 μM, 8.1 ± 0.9 μM, and 1.3 ± 0.3 μg/ml, respectively. Binding was not blocked by the control peptide of GRGESPA or the anti-GPIb monoclonal antibody (LJ-1b1) (Table II).

Previous study from an independent laboratory showed that fibronectin did not inhibit binding of fibrinogen to thrombin-stimulated platelets (14). The 29-kDa fragment inhibited the binding of fibrinogen to platelets stimulated by thrombin or by ADP (Fig. 2).

**ADP-induced Aggregation**—The 29-kDa dispase fragment inhibited the ADP-induced aggregation of platelets in a dose-dependent manner. Inhibition of approximately 80% was achieved at a concentration of 2.1 μM of this fragment. Native fibronectin and the GRGDSPA peptide also inhibited the ADP-induced aggregation of platelets in a dose-dependent manner (Fig. 3).

**Direct Binding of the 29-kDa Fragment to Platelets**—Direct binding of the 29-kDa fragment to thrombin-stimulated platelets was saturable with a B_{max} of 98,000 ± 4,600 sites/platelet (K_d = 4.6 ± 0.5 x 10^{-6}M) (Fig. 4). The binding was inhibited by the unlabeled 29-kDa fragment and native fibronectin but not by the GRGDSPA peptide or the monoclonal anti-GPIIb-IIIa antibody (LJ-CP8) (Table III).

**Interaction of the 29-kDa Dispase Fragment of Fibronectin with Immobilized GPIIb-IIIa**—Selected polyclonal antibody (FK-2) recognized only fibronectin and did not react with fibrinogen or von Willebrand factor (19). This antibody also reacted with the 29-kDa fragment of fibronectin (Fig. 5A). This finding indicates that the epitope recognized by FK-2 is located within this fragment. We then evaluated the interaction of the fragment with immobilized GPIIb-IIIa using enzyme-linked immunosorbent assay. The 29-kDa fragment interacted very well with immobilized GPIIb-IIIa (Fig. 5B); however, as would be expected, the fragment failed to react with immobilized GPIb.

**DISCUSSION**

RGD sequence, or closely related sequences, is a major binding site of fibronectin to activated platelets (34) and interacts directly with glycoprotein IIIa (35). A study of unstimulated platelets showed GP Ib/IIa to be a fibronectin receptor. RGD-containing peptides also block the binding of fibronectin to unstimulated platelets (36). These indicate that RGD-containing ligands play a central role in the binding of fibronectin to stimulated and unstimulated platelets.

Recently, additional binding site(s) in fibronectin to GPIIb-IIIa have been located in fibronectin (residues 1359-1436) that lacks the RGD sequence (15). This result implies that multiple sites in fibronectin might be involved in its recognition by GPIIb-IIIa. In this paper, we demonstrated that a 29-kDa dispase fragment of fibronectin that lacks the RGD sequence inhibited binding of fibronectin to thrombin-stimulated platelets as well as the ADP-induced aggregation of platelets. This fragment bound directly to thrombin-stimulated platelets. In addition, this fragment reacted with the immobilized GPIIb-IIIa. We thus conclude that fibronectin contains RGD-independent binding domain(s) to the receptor on activated platelets, probably GPIIb-IIIa. This binding site is distinct from the one identified by Bowditch et al. (15).
If, as it appears, the RGD-independent binding domain to platelets is present, the role of this binding should be identified. The present study showed that the 29-kDa dispase fragment of fibronectin inhibited the platelet aggregation induced by ADP. As an agonist, ADP induces fibronectin receptors but not fibronectin receptors, whereas thrombin activates both (6, 37). Our platelet aggregation study indicates that the 29-kDa fragment can bind to fibronectin receptors as well as to fibronectin receptors. The inhibition of fibronectin binding by the 29-kDa fragment also provides evidence that this fragment binds to the fibrinogen receptor. These results suggest that the RGD-independent binding domain(s) of the 29-kDa fragment modulates platelet aggregation.

The 29-kDa fragment reacted with immobilized GPIIb-IIIa, which indicates that the receptor of the RGD-independent domain(s) of fibronectin is GPIIb-IIIa. However, the GRGDSF peptide and the monoclonal anti-GPIIb-IIIa antibody, which inhibit the binding of fibronectin to GPIIb-IIIa (15) as well as that of fibronectin to platelets, do not block the direct binding of the 29-kDa fragment to platelets. Evidence strongly suggests that the binding site(s) of the 29-kDa fragment on the receptor is distinct from that of RGD sequence and the sequence corresponding to residues 1359-1436. Fibronectin may prevent thrombus formation by interfering with the interaction of fibrinogen with GPIIb-IIIa via an RGD-independent manner as well as an RGD-dependent manner. These findings are clues to the chemical basis of the involvement of fibronectin in the formation of platelet thrombus.

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