

Binding of Fibronectin to α -Granule-deficient Platelets

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ABSTRACT Most of the proposed functions for fibronectin involve its interaction with cells, yet the molecular nature of cellular fibronectin binding site(s) has remained obscure. Thrombin induces saturable platelet binding sites for plasma fibronectin and concurrently stimulates surface expression of a number of platelet α -granule constituents including thrombospondin and fibrin which are known to interact with fibronectin. To test the hypothesis that these (or other α -granule proteins) mediate plasma fibronectin binding, we used platelets of patients with the Gray Platelet Syndrome. These cells were deficient in thrombospondin, β -thromboglobulin, platelet factor 4, fibronectin, and fibrinogen as measured in radioimmunoassay. They also had reduced von Willebrand factor content as judged by immunofluorescence. At plasma fibronectin inputs from 0.03 to 3 times the apparent kilodalton, these Gray platelets bound virtually identical quantities of fibronectin as normal cells. Thus, platelets containing 1,500 molecules of thrombospondin per platelet could bind more than 100,000 molecules of plasma fibronectin per cell following thrombin stimulation. These data preclude any simple model in which newly surface expressed thrombospondin (or other α -granule protein) functions as the major thrombin-stimulated plasma fibronectin receptor in this cell type.

Fibronectins have been implicated in a number of cellular interactions including adhesion to fibrin and to collagen, opsonization, and in the regulation of cell migration (1, 2). Each of these potential functions of fibronectin requires that it bind to cell surfaces. Although both glycolipids (3) and protein constituents (4) have been implicated as cellular fibronectin "receptors" the molecular identity of such "receptors" has remained obscure. We recently described inducible, specific, and saturable binding of soluble plasma fibronectin to thrombin-stimulated platelets. This interaction has an apparent dissociation constant of 3×10^{-7} M (5), approximately half the normal plasma fibronectin concentration (1), and 120,000 molecules per platelet are bound at saturation.

While thrombin supports this interaction, ADP and epinephrine, which do not induce secretion under our assay conditions, failed to support plasma fibronectin binding (5). In addition to secretion, thrombin also stimulates appearance of certain constituents of platelet α -granules on the cell surface (6-9). Thus, the possibility that a newly surface expressed α -granule constituent mediates plasma fibronectin attachment to thrombin-stimulated platelets merits consideration.

One candidate α -granule protein is platelet fibrinogen, or platelet fibrin, which is present at a level of approximately 100,000 molecules per platelet and is expressed on the cell surface following thrombin stimulation (8). Moreover, fibrinogen and fibrin interact with fibronectin, although the non-covalent interaction is of low affinity at 37°C (10). Neverthe-

less, platelet fibrinogen or fibrin appears unlikely to serve such a role, since we recently found that platelets from afibrinogenic patients, containing <2% of the normal fibrinogen content, bind plasma fibronectin normally (11).

A second candidate, α -granule protein for such a function is thrombospondin (TSP). This protein, purified in native form by Lawler et al. (12), is a trimer of 150-kdalton subunits and approximately 40,000 molecules are present per platelet (13). Thus, 120,000 molecules of TSP subunit per platelet could accommodate fibronectin binding, in a 1:1 stoichiometry. TSP is expressed on the surface of thrombin-stimulated cells (6), and has been reported to bind both fibrinogen and fibronectin (14). Moreover, since TSP is synthesized by fibroblasts (15), endothelial cells (16, 17), and smooth muscle cells (15), and appears to be a matrix protein (15), it might support fibronectin interactions with cells other than platelets. Recent studies of Lahav et al. (18) have shown that when fibronectin derivatized with *N*-succinimidyl-3-[2-(nitro-4-azidophenyl)-2-aminoethylthio] propionate is reacted with adherent platelets, photoactivation results in cross-linking to thrombospondin. Thus, TSP might serve as the newly expressed plasma fibronectin receptor on thrombin-stimulated platelets.

The Gray Platelet Syndrome is a mild bleeding diathesis in which platelets have a markedly reduced content of alpha granules (19) and are deficient in a variety of α -granule proteins, including fibrinogen, platelet factor 4, β -thromboglobulin (19), fibronectin, and von Willebrand factor (20).

They also show reduced staining of a band of the mobility of TSP on SDS PAGE (19). Our investigation's purpose was to test the potential role of TSP (or other α -granule proteins) in the binding of plasma fibronectin to thrombin-stimulated platelets by analysis of its interaction with platelets of patients with the Gray Platelet Syndrome.

MATERIALS AND METHODS

Patients: The two patients with Gray Platelet Syndrome are unrelated individuals who are followed at the University of Minnesota and have been extensively characterized in published literature (19). Their platelets have been reported to have <10% of the normal content of α -granules as assayed by morphometric techniques, and <10% of the normal content of platelet factor 4, β -thromboglobulin, and fibrinogen (19).

Platelet Isolation and Binding Assays: Anti-coagulated blood from the gray platelet patients and normals were simultaneously obtained in acid citrate dextrose anticoagulant at the University of Minnesota. Platelet-rich plasma was isolated by differential centrifugation as described (5), and the platelet-rich plasma was shipped Air Freight to San Diego, where washed platelets were prepared and studied within 10 h of venipuncture. The technique of platelet washing has been described in detail (5). Briefly, platelet pellets obtained by centrifugation of platelet-rich plasma, were resuspended in 2 ml of modified Tyrode's buffer, pH 6.5, containing 2 mM MgCl₂ and 0.1% BSA, and gel filtered on the column (2.5 × 10 cm) to Sepharose 2B equilibrated in the modified Tyrode's buffer at pH 7.4. In binding assays, 10⁸ platelets per milliliter were incubated in the presence of 2 U/ml purified human α -thrombin (gift of Dr. John Fenton, New York State Department of Health) and of various doses of ¹²⁵I-labeled fibronectin (purification and radiolabeling described in reference 5) for 30 min at 37°C, and triplicate 50- μ l aliquots were layered onto 200 μ l of 20% sucrose in modified Tyrode's buffers and centrifuged for 3 min in a Beckman microfuge B (Beckman Instruments, Inc., Palo Alto, CA). Tips were amputated and counted, and the fibronectin bound was calculated from the specific activity of ligand using a molecular weight of 440,000.

Radioimmunoassay: 10⁹ platelets per milliliter were lysed in a buffer containing 1% Triton X-100, snap frozen, and stored in aliquots at -70°C. Each aliquot was thawed only once prior to analysis. Radioimmunoassays for platelet factor 4 (21), fibrinogen (22), and fibronectin (23) have been described in detail previously. Radioimmunoassay for β -thromboglobulin employed antibody and antigen generously provided by Dr. Duncan S. Pepper of the Scottish Transfusion Service, Edinburgh, U.K. It was radiolabeled, and the assay was performed using ammonium sulfate precipitation employing the identical buffers used in the platelet factor 4 assay (21). The radioimmunoassay for thrombospondin has been described in detail elsewhere (U. Marzec, L. Harker, and M. Ginsberg, manuscript in preparation). Briefly, human TSP was purified from a releasate of thrombin-stimulated platelets by a modification of the procedure of Lawler et al. (12), the purified antigen was >95% homogeneous as judged by PAGE in the presence of SDS. It was radiolabeled with ¹²⁵I employing chloramine T oxidation to a specific activity of 1 μ Ci/ μ g. Anti-TSP was raised in rabbits, and the antibody was detected by its ability to bind ¹²⁵I-TSP in the radioimmunoassay. This antibody was characterized as immunoprecipitating only TSP from radiolabeled platelet lysates and did not bind to fibronectin or fibrinogen in their radioimmunoassays. The TSP radioimmunoassay was performed using buffer systems described for the platelet factor 4 radioimmunoassay (21) and a double antibody immunoprecipitation. Assay sensitivity was ~1 ng of TSP/ml and was not influenced by the presence of

plasma, platelet lysates, or heparin (U. Marzec, L. Harker, and M. Ginsberg, manuscript in preparation).

Immunofluorescent Staining: Immunofluorescence protocols have been published in detail elsewhere (24). Briefly, platelets were fixed in suspension with 2% paraformaldehyde for 1 h in melting ice. The paraformaldehyde was neutralized with 20 mM ammonium chloride in 10 mM Tris, 150 mM NaCl, pH 7.4, and the cells were washed in 10 mM Tris 150 mM NaCl, pH 7.4, and permitted to settle onto polylysine-coated cover slips. Following this, they were permeabilized by 3-min treatment in 0.1% Triton X-100, and stained using rabbit anti-TSP, anti- β -thromboglobulin, and anti-platelet factor 4, anti-von Willebrand factor, or antifibronectin, followed by fluoresceinated goat anti-rabbit immunoglobulin and rhodaminated goat anti-human fibrinogen. The characterization of all the primary antibodies is reported elsewhere (25), as are the filter systems employed. Results were recorded on Tri-X panchromatic film.

RESULTS AND DISCUSSION

Reduction of α -Granule Constituents in Gray Platelets

To confirm that the platelets of the two patients studied were deficient in the relevant proteins at the time of study, lysates of their cells were analyzed by radioimmunoassay. The gray platelets were profoundly deficient in TSP, platelet factor 4, and β -thromboglobulin (content ~5% of normals) (Table I). Fibrinogen and fibronectin were also reduced but to a lesser degree (10% and 25% of normal, respectively). Since plasma fibrinogen (gray = 1,850 μ g/ml; normal = 2,500 μ g/ml) and fibronectin (gray = 418 μ g/ml; normal = 418 μ g/ml) were not reduced in the gray platelet patients, the comparative abundance of these proteins in gray platelet lysate may be due to plasma contamination. In any event, with respect to TSP, the gray platelets contained an average of 1,500 molecules per platelet, indicating reduced content of this protein.

We considered the possibility that TSP antigen (or other proteins) was destroyed in the gray platelets during lysis. To test this, we performed immunofluorescence analysis of the

TABLE I

Content of α -Granule Proteins in Normal and Gray Platelets

	Normal platelet content (μ g/10 ⁹ platelets)	Gray Platelet content (μ g/10 ⁹ platelets)
TSP	24.6	1.0
Fibrinogen	88.3	6.5
Fibronectin	2.5	0.6
Platelet factor 4	9.5	0.5
β -TSP	18.0	0.7

1% Triton-X-100 lysates, derived from platelet suspensions containing 10⁹ platelets per milliliter, were assayed for each protein in radioimmunoassays as described in Materials and Methods. Results are the means of determinations on two different Gray Platelet patients and simultaneously prepared and assayed normal subjects.

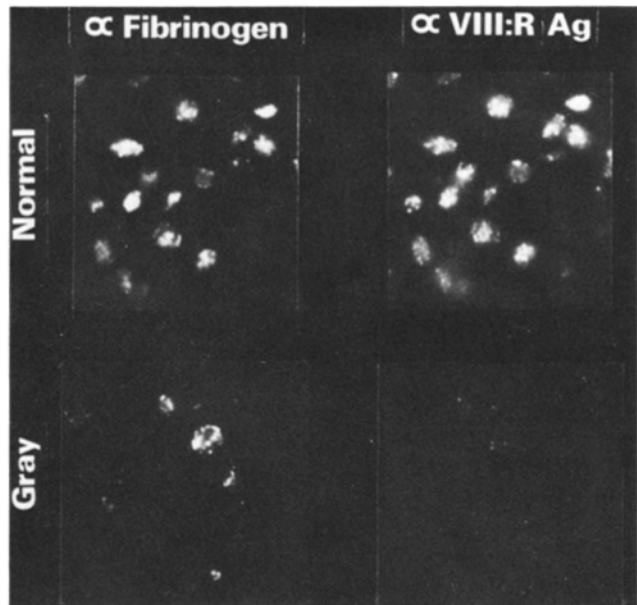


FIGURE 1 Reduced immunofluorescent staining for fibrinogen and von Willebrand Factor in gray platelets. Platelets were double stained for fibrinogen and von Willebrand Factor as described in Materials and Methods. The left panels illustrate fibrinogen staining and the right illustrate von Willebrand Factor staining. The normal cells are depicted in the upper panel and the gray platelets below. × 1,600.

unextracted cells and confirmed the deficiency of TSP and other α -granular proteins in the gray cells (not shown). In addition, the gray platelets did not stain for Factor VIII von Willebrand factor (Fig. 1).

Binding of Plasma Fibronectin to Thrombin-stimulated Gray Platelets

To evaluate their ability to bind plasma fibronectin, the gray platelets were incubated with various doses of ^{125}I -fibronectin in the presence of 2 U of thrombin/ml, and bound ligand was separated from free by centrifugation through 20% sucrose. TSP-deficient platelets and normal platelets bound virtually identical quantities of fibronectin at input doses from 0.03 to 3 times the apparent kilodalton, 3×10^{-7} M (5) (Fig. 2).

Thus, platelets containing <5% of the normal TSP content bound plasma fibronectin as well as normal cells. This result suggests that if TSP participates in thrombin-stimulated plasma fibronectin binding, it is normally present in at least 20-fold excess of the quantity required for this function. Secondly, since platelets containing only 1,500 molecules of TSP per platelet could bind more than 100,000 molecules of plasma fibronectin per platelet, TSP must not be binding fibronectin to the platelet surface in a one-to-one stoichiometry. These data thus preclude any simple model in which

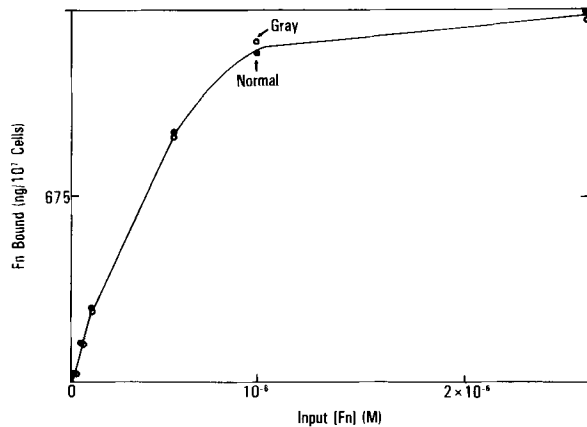


FIGURE 2 Binding of plasma fibronectin to normal and gray platelets. ^{125}I -fibronectin at the indicated input concentrations was incubated at 37°C with 10^8 platelets/ml in the presence of 2 U thrombin/ml for 30 min. Under conditions which inhibit TSP re-binding, similar doses of thrombin induce secretion of virtually all platelet thrombospondin (8). Binding was measured as described in Materials and Methods. (●) Normal. (○) Gray platelets. Means of triplicate determinations on two different Gray Platelet patients and two simultaneously assayed normals.

surface expression of TSP accounts for plasma fibronectin binding to suspensions of thrombin-stimulated platelets.

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