Identification of a New Class of Inducible Receptors on Platelets

Thrombospondin Interacts with Platelets via a GPIIb-IIIa-independent Mechanism

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Abstract

Thrombospondin with fibrinogen, fibronectin, and von Willebrand factor binds to platelets stimulated with agonists and support platelet adhesive functions. The receptors for the latter three proteins are associated with membrane glycoprotein GPIIb-IIIa. Thrombasthenic platelets deficient in GPIIb-IIIa have been utilized to examine the role of this membrane protein in the interactions of thrombospondin with platelets. Radioiodinated thrombospondin bound to thrombin-stimulated platelets from normal and thrombasthenic donors with a similar affinity and capacity. As monitored with a monoclonal antibody to thrombospondin, the divalent ion-dependent and -independent pathways for the expression of the endogenous pool of thrombospondin on the surface of thrombin-stimulated platelets from normal and thrombasthenic donors were also qualitatively and quantitatively similar. GPIIb-IIIa or ligands associated with GPIIb-IIIa thus are not essential for the binding of thrombospondin to platelets. Therefore, thrombospondin interacts with unique receptors on platelets.

Introduction

Fibrinogen (Fg), fibronectin (Fn), and von Willebrand factor (vWF) are large, multimeric glycoproteins that regulate platelet adhesive functions by interacting with specific receptors on the cell surface. Receptors for Fg, Fn, and vWF are not available on resting platelets but are rapidly induced by agonists such as thrombin (reviewed in ref. 1). A single platelet membrane glycoprotein, GPIIb-IIIa, is an obligate component of the inducible receptor for these adhesive proteins. The deficit in Fg (2, 3), Fn (4), and vWF (5) binding to thrombin-stimulated thrombasthenic platelets, which are genetically deficient in GPIIb-IIIa, was among the first evidence implicating GPIIb-IIIa as an inducible receptor for these adhesive proteins.

Thrombospondin (TSP) is a fourth platelet adhesive protein. This classification is suggested by its similarity in physicochemical properties to the other adhesive proteins (1, 6), by its capacity to agglutinate platelets and platelet membranes (7-9), and by the inhibition of platelet aggregation by polyclonal (10, 11) and monoclonal antibodies (12) to TSP. TSP also interacts with a stimulus-induced set of saturable binding sites on platelets (13). Thrombin supports the binding of TSP to 35,100±9,600 sites/platelet with a dissociation constant (Kd) of 250 nM (13). Additional common properties of the interactions of TSP, Fg, Fn, and vWF with thrombin-stimulated platelets include a requirement for divalent ions and the inhibition of these interactions by Fg (13) and certain monoclonal antibodies to GPIIb-IIIa (13, 14). The inhibition of TSP binding by Fg and these antibody reagents suggests that GPIIb-IIIa, and more specifically, Fg bound to or the Fg receptor on GPIIb-IIIa, directly mediates or is proximal to the TSP binding site. The interaction of TSP with platelets is distinct from that of the other adhesive proteins in two respects. Firstly, TSP also binds to a second set of sites available on resting platelets (Kd, 50 nM; 3,100±1,000 molecules/platelet) in a divalent ion independent reaction (13). Secondly, in contrast to Fg, Fn, and vWF, plasma does not constitute a significant source of TSP. Therefore, the predominant mechanism for surface expression of TSP is dependent upon secretion of TSP from its intracellular pool within a-granules (14-17).

In this study, the role of GPIIb-IIIa in the interactions of TSP with platelets has been examined. To assess this question, the binding of exogenous TSP to and the surface expression of endogenous TSP on thrombasthenic platelets deficient in GPIIb-IIIa has been analyzed. The results indicate that TSP interacts with a unique set of inducible receptors, independent of GPIIb-IIIa and Fg.

Methods

Platelets. Platelets were isolated by differential centrifugation followed by gel filtration on Sepharose 2B in divalent ion-free or calcium-free Tyrode's buffer, pH 7.3, containing 0.1-2% bovine serum albumin (13, 14, 17). During the course of this study, two thrombasthenic patients were studied on three separate occasions. These thrombasthenic patients were made available by Dr. Margaret Johnson of the University of Delaware. Their deficit in adhesive protein binding and GPIIb-IIIa has been extensively documented in the literature (e.g., 2, 4, 18) while the levels of other membrane glycoproteins (GPI and GPIV) have been reported to be normal (19). On two of the occasions, monoclonal antibodies to GPIIb-IIIa were used to verify the deficiency of the membrane glycoprotein. The deficit in GPIIb-IIIa ranged from 85 to 87% as measured with the complex specific antibody AP2, kindly provided by Dr. Thomas Kunicki (20), and 65-85% as measured with the GPIIb monoclonal antibody PM-1 (21). The platelet-rich plasma from the thrombasthenic patients as well as from a normal donor drawn in parallel were shipped at 22°C and analyzed within 24 h. Under these conditions, the binding of Fg, Fn, and vWF to freshly isolated and the shipped normal platelets were found to be very similar (4, 21, and unpublished observations).

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Received for publication 22 July 1986.

1. Abbreviations used in this paper: GP, glycoprotein; Fg, fibrinogen; Fn, fibronectin; TSP, thrombospondin; vWF, von Willebrand factor.

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0021-9738/86/12/1713/04 $1.00
Volume 78, December 1986, 1713-1716
Platelet binding and surface expression of TSP. TSP was isolated from fresh human platelets according to the method of Lawler et al. (22) with the modifications described from our laboratory (23). The product was radiiodinated to specific activities of 0.5–1.0 μCi/μg by a modified chloramine T procedure, and 125I-TSP ligands utilized in this study exhibited the characteristics previously described (13). Varying concentrations of TSP ranging from 0.01 to 0.7 μM were added to nonstimulated or thrombin-stimulated (0.5–1.0 U thrombin/ml) platelets at 2 × 10^8/ml (counted electronically in a ZF Coulter counter, Coulter Instruments, Inc., Hialeah, FL) in Tyrode’s-albumin buffer containing the specified divalent ions. Binding was measured after a 30-min incubation at 37°C by centrifugation of triplicate 50-μl aliquots of the reaction mixtures through 20% sucrose (4, 13, 14).

Surface expression of endogenous TSP was monitored using the monoclonal antibody TSP-1-1 (17). The antibody was purified from ascitic fluid on protein A Sepharose and radiiodinated to specific activities of 0.5–2.0 μCi/μg. Binding of 125I-TSP-1-1 to thrombin-stimulated platelets was measured by adding varying concentrations of the antibody in the 0.1–0.8 μM range to platelets at 2 × 10^8/ml. Conditions of these assays and for separation of cell-bound from free ligand were the same as for 125I-TSP, except that incubations were performed for 60 min at 22°C (conditions optimal for maximal surface expression of TSP with the antibody reagent [17]).

The data for the binding of 125I-TSP and 125I-TSP-1-1 to the normal and thrombasthenic platelets were analyzed in the Ligand computer program (24) as previously described (13). Accordingly, the total binding data was analyzed to derive a value for the nonsaturable binding, Nf, in the analyses shown, Nf for TSP binding ranged from 0.07 to 0.4% of the TSP input concentration. The values of Nf for TSP-1-1 ranged from 0 to 0.02%. These values, the percentage of total input concentration of TSP or TSP-1-1, were consistent with previously reported values and with experimentally determined levels of nonsaturable binding. Saturable binding was then derived as the difference between the levels of total binding and nonsaturable binding and was used to estimate the apparently dissociation constants and number of binding sites per platelet.

Results

The interaction of exogenously added TSP with thrombasthenic and normal platelets was compared. Varying concentrations of TSP were added to thrombin-stimulated platelets, and binding was measured after 30 min at 37°C. The binding of TSP to the platelets from one of the two thrombasthenic patients is shown in Fig. 1. The binding data provides evidence for saturable and high capacity binding of TSP to thrombasthenic platelets. Nonsaturable binding estimated in the Ligand program was 0.04% of the total TSP concentration added, and this value was used to derive the saturable binding curve shown in Fig. 1. Similar analyses were performed with platelets from the second thrombasthenic patient and the normal donor drawn in parallel. The binding parameters obtained from Scatchard plots of the data have been summarized in Table I. The apparent dissociation constants, Kd, quantitated in this study for TSP binding to the two thrombasthenic patients and the normal donor are very similar and fall within the normal range (13). In other experiments performed during the course of this study using normal platelets (n = 3), the Kd for TSP binding ranged from 200 to 500 nM and the number of TSP binding sites ranged from 11,700 to 45,000. There is thus no deficit in the number or affinity of TSP binding sites on thrombin-stimulated thrombasthenic platelets.

TSP also interacts with saturable binding sites on nonstimulated platelets via a divalent ion–dependent reaction. Although this interaction is unlikely to be dependent upon GP IIb-IIIa based on the failure of Fg and monoclonal antibodies to GPIIb-IIIa to inhibit binding and the inappropriate stoichiometry between these TSP sites (3,000 ± 1,000 sites/platelets [13]) and GP IIb-IIIa (~50,000 sites/platelet [20]), direct verification of this conclusion was sought. 125I-TSP at 50 nM, its determined Kd for resting platelets (13), was added to nonstimulated normal or thrombasthenic platelets, and binding was measured after a 30-min incubation at 37°C in the presence of 5 mM EDTA. The number of TSP molecules bound per platelet was: 1,400 for thrombasthenic patient A; 3,300 for thrombasthenic patient B; and 1,700 for the normal donor. There is thus no deficit in the binding of TSP to nonstimulated as well as to thrombin-stimulated thrombasthenic platelets.

Monoclonal antibody TSP-1-1 was used to monitor expression of the endogenous pool of TSP on the surface of thrombasthenic and normal platelets. As shown in Fig. 2, TSP is expressed on the surface of thrombin-stimulated thrombasthenic

![Figure 1](image_url)

**Figure 1.** Binding of exogenously added TSP to thrombin-stimulated thrombasthenic platelets. Varying concentrations of TSP and a constant amount of tracer 125I-TSP were added to platelets from thrombasthenic patient B (2 × 10^8/ml) in Tyrode’s-albumin buffer containing 1 mM Ca and Mg. Platelets were stimulated with 1 U/ml thrombin, and binding was measured after a 30-min incubation at 37°C. The total binding (●) was used to estimate the nonsaturable binding (□) in the Ligand computer program and subtracted from the total binding to derive the saturable binding isotherm (○).

<table>
<thead>
<tr>
<th>Binding parameter</th>
<th>Platelet donor</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Kd, nM</td>
<td>325</td>
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<tr>
<td>Maximal TSP binding (molecule/platelet)</td>
<td>15,000</td>
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Varying concentrations of TSP were added to the isolated normal or thrombasthenic platelets at 2 × 10^8/ml in Tyrode’s-albumin buffer containing 1 mM calcium and magnesium. The platelets were stimulated with 1 U/ml thrombin, and binding was measured after 30 min at 37°C. The binding parameters were derived from Scatchard plots of the data generated by the Ligand computer program.
platelets. Total binding of $^{125}$I-TSP-1 to the platelets of thrombasthenic patient B and the normal donor isolated in parallel is shown. As $N_1$ was estimated to be 0–0.2% of the total binding, the data also correspond closely to the saturable binding. The $K_d$ of the antibody was estimated to be 0.17 μM for the normal donor and 0.2 μM for the thrombasthenic patient. Consistent with the high binding of exogenously added TSP to the platelets from thrombasthenic patient B, the maximum number of TSP-1 binding sites was also high as determined from Scatchard plots of the data, 19,350 vs. 17,000 for the normal donor. While the stoichiometry of TSP-1 binding to TSP on the platelet surface was not measured directly, others (25) have reported that monoclonal antibodies to the heparin binding region interact with TSP in solution with a 1:1 stoichiometry although TSP is comprised of three apparently identical subunits (22). The divalent ion independent mechanism of TSP surface expression was also found to be fully functional on the thrombasthenic platelets. With $^{125}$I-TSP-1 added at 0.5 μM, the thrombin-stimulated platelets from the normal and thrombasthenic patient bound 2,400 and 3,700 antibody molecules/platelet, respectively, in the presence of 5 mM EDTA.

Discussion

The results of this study indicate that thrombasthenic platelets deficient in GPIIb-IIIa can interact with TSP by the same mechanisms and with similar binding characteristics with respect to affinity and number of sites as observed with normal platelets. This conclusion applies to the binding of exogenously added TSP, which may occur when platelets interact with other cells or cell matrices containing TSP. It also applies to the expression of endogenous TSP on the platelet surface, presumably the predominant mechanism for cell surface expression of TSP on platelets. The results do not indicate that TSP–platelet interactions are necessarily normal with thrombasthenic platelets.

Thrombasthenia is heterogeneous (26), and Hourdille et al. (27) have reported a decrease in TSP surface expression on thrombasthenic platelets. Regardless of these differences, our data clearly demonstrate that GPIIb-IIIa is not necessary for the various pathways of TSP interactions with platelets.

We have previously shown that Fg and monoclonal antibodies to GPIIb-IIIa inhibit the binding of exogenous TSP to platelets (13, 14). In view of the present data, a relationship but not an identity between the TSP receptors, GPIIb-IIIa, and the binding sites for the other adhesive proteins on GPIIb-IIIa must be postulated. The relationship between these adhesive protein receptors may be due to their close proximity as suggested by Asch et al. (28) or they could be allosterically linked. This conclusion suggests that thrombin not only converts GPIIb-IIIa from a nonfunctional to a functional receptor for Fg, Fn, and vWF but also induces a previously unrecognized receptor, unrelated to GPIIb-IIIa, the TSP divalent ion–dependent binding site. The data also indicate that the presence of binding sites for other adhesive proteins is not an obligate requirement for TSP binding. In addition, although TSP and Fg interact with high affinity (29), TSP binds normally to the thrombasthenic patients that lack not only GPIIb-IIIa and the Fg receptor but also platelet Fg per se (4). Neither the affinity nor the extent of the measured interactions of TSP with platelets thus are absolutely dependent upon Fg. Finally, the hemostatic problems of thrombasthenic patients are extensive. TSP thus cannot be sufficient to support the adhesive reactions of platelets necessary for hemostasis. This conclusion does not exclude an auxiliary role for TSP in platelet function such as in stabilizing formed platelet aggregates (10).

Acknowledgments

We thank Dr. Margaret Johnson for providing access to the thrombasthenic patients. The monoclonal antibody, AP2, was generously provided by Dr. Thomas Kunicki. The technical assistance of Roger Wolf, Jane Forsyth, and Vicky Byers-Ward are gratefully acknowledged. We thank Judy Taylor for the preparation of the manuscript.

Dr. Aiken was supported by NIH training grant AI07244. This work was supported by NIH grant HL-16411 and HL-28235. This is publication number 4467 from the Department of Immunology of the Research Institute of Scripps Clinic.

References


Figure 2. Surface expression of TSP on thrombin-stimulated thrombasthenic and normal platelets. Normal and thrombasthenic platelets from patient B at 2 × 10⁹/ml were stimulated with 0.5 U/ml thrombin in Tyrode’s–albumin buffer containing 2 mM Ca and Mg. Varying concentrations of monoclonal antibody TSP-1 in radiolabeled form were added to the platelets, and binding was measured after a 60-min incubation at 22°C.


