The Interaction of Thrombospondin with Platelet Glycoprotein GPIIb-IIIa *

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The interaction of human platelet thrombospondin (TSP) with human platelet glycoproteins GPIIb-IIIa was studied using a solid-phase binding assay. Polystyrene test tubes were coated with TSP, and 125I-labeled GPIIb-IIIa was added, allowed to bind, and the bound radioactivity was measured. After 90 min, the binding became time independent, and in most experiments, more than 10% of the exogenously added radioactivity was bound to the tube. Analysis of the bound radioactivity by polyacrylamide gel electrophoresis and autography indicated that it was from labeled GPIIb-IIIa. Several lines of evidence indicate that the binding of GPIIb-IIIa to TSP was specific. (a) TSP immobilized on plastic or Sepharose bound 3-10-fold more GPIIb-IIIa than immobilized bovine serum albumin. (b) Addition of unlabeled excess GPIIb-IIIa, reversed the binding of 125I-labeled GPIIb-IIIa to immobilized TSP. (c) Addition of EDTA inhibited the binding of GPIIb-IIIa to TSP by more than 90%, whereas addition of 1 mM CaCl₂ and 1 mM MgCl₂ potentiated the binding by more than 100%. (d) Monoclonal antibodies against TSP and GPIIb-IIIa inhibited the binding by 30-70% as compared with control and polyclonal anti-fibrinogen antisera. (e) A plot of GPIIb-IIIa bound versus GPIIb-IIIa added was best described as a rectangular hyperbola by regression analysis with half-saturation at 60 ng/ml GPIIb-IIIa. Similar results were obtained when labeled TSP was added to tubes coated with GPIIb-IIIa. These results show that TSP and GPIIb-IIIa can specifically interact in vitro and suggest that GPIIb-IIIa may function as a platelet TSP receptor during platelet aggregation.

When activated by such physiological agonists as thrombin and ADP, human blood platelets secrete a large glycoprotein, now generally referred to as thrombospondin (TSP), which is an M₅, 450,000 glycoprotein composed of three identical intermolecular disulfide-linked polypeptide chains that migrate on discontinuous SDS gels as a single band of 180,000 daltons.

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§ The abbreviations used are: TSP, thrombospondin; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxycylethenenitrilo)]tetraacetic acid.

1. TSP is a major platelet-secreted protein accounting for more than 25% of the total protein secreted by activated platelets (2). Much information is known about the physicochemical properties of TSP. For example, TSP, like fibronectin, contains distinct structural domains that, when cleaved from the intact molecule by proteolytic enzymes, still maintain their ligand-binding properties. These TSP fragments have been shown to interact with heparin (9), fibrinogen (2), and type V collagen (4). The structure of the intact molecule as revealed by electron microscopy consists of several globular regions connected by fibronectin-like domains that resemble a South American bola, a weapon made of long cord with heavy balls at the end, used for roping cattle.

Platelet aggregation is an important step in the hemostatic process. As a consequence of vessel injury, platelets become activated, aggregate, and, in concert with the plasma-clotting system, seal the damaged vessel. The importance of platelets in normal hemostasis can be appreciated when patients who develop thrombocytopenia run the risk of severe hemorrhage. Therefore, an understanding of how platelets and their associated proteins function in blood coagulation is critical to the development of antithrombotic therapies.

The role of thrombospondin in the mechanism of platelet aggregation and blood coagulation is poorly understood. It is widely believed that TSP functions to cross-link platelet aggregation irreversibly during the secretion phase of platelet aggregation. This conclusion is based on several experimental observations. (a) At least 80% of the total TSP secreted by platelets in response to thrombin, a potent aggregating agent, binds the platelet surface (5). (b) TSP binds fibrinogen (2). (c) Antibodies against the platelet fibrinogen receptor, GPIIb-IIIa, inhibit the binding of exogenously added TSP to activated platelets (6). (d) TSP promotes platelet aggregation (7). (e) Antibodies against TSP inhibit platelet aggregation (7, 8). (f) TSP co-localizes with fibrinogen and GPIIb-IIIa on the surface of activated platelets (9).

One interpretation of the studies described above suggests that during platelet aggregation, TSP and fibrinogen share the same platelet receptor. To test this hypothesis, we developed an in vitro binding assay to measure the interaction of TSP with the platelet fibrinogen receptor, GPIIb-IIIa. Our results indicate that TSP specifically and saturably binds the GPIIb-IIIa complex.

EXPERIMENTAL PROCEDURES

Materials—All reagents, unless specified otherwise, were purchased from Sigma. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were obtained from Bio-Rad. Iodine beads were purchased from Pierce Chemical Co. Na₂O₁₇I was purchased from Du Pont-New England Nuclear. The monoclonal antibodies A₆₄ and SSA to the GPIIb-IIIa complex were gifts of Dr. Joel Bennett (University of Pennsylvania) and were stored in a 10.9 mg/ml stock.
solution in phosphate-buffered saline. Monoclonal anti-TSP was characterized previously (7). AAs is directed against the GPIIb-III complex (10), and SSAs is GPIII specific (11). TSP was purified by fibrinogen-Sepharose chromatography from the released proteins of ionophore-stimulated platelets essentially as described previously (2).

Preparations of GPIIb-III, contained no GPIV as indicated by the absence of cross-reactivity with anti-GPIV antibody, kindly provided by Dr. Tandon of the American Red Cross, Rockville, MD. Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described previously (12). Preparations of GPIIb-III, were purified by a three-step procedure consisting of Lens culinaris affinity chromatography, anion-exchange chromatography, and anti-GPIIb-III (AAAs) monoclonal antibody affinity chromatography as described previously (12).

RESULTS

Binding of GPIIb-III to Immobilized TSP—In 1 h, approximately 1.5 ng of GPIIb-III bound to 1 µg of immobilized TSP as calculated from the specific activity of the [125I]GPIIb-III, added to the incubation mixture, whereas less than 0.10 ng bound in the presence of unlabeled excess GPIIb-III, during the same period of time (Fig. 1). The binding of GPIIb-III, to TSP became time independent after 40 min and was partially reversible with excess unlabeled GPIIb-III, added 30 min after initiation of binding (Fig. 1). These results indicate that the bound radioactivity represented binding buffer containing 100 ng of protein in the presence of 50 µg of unlabeled GPIIb-III, (open triangles) or in the absence of unlabeled GPIIb-III, (closed circles) was added to polystyrene tubes coated with TSP. After 1 h at room temperature, the tubes were washed and counted, and the amount of protein bound was calculated from the specific activity of the [125I]GPIIb-III, added. Additionally, in a separate experiment, 50 µg of unlabeled GPIIb-III, was added to the reaction mixture after 30 min (open circles) and the bound [125I]GPIIb-III, measured after 40, 50, and 60 min of incubation. The data points are the mean of triplicate determinations, and the bars represent the standard deviation.

To establish that the bound radioactivity represented GPIII, and not some labeled impurity, tubes were eluted with SDS and the eluted material analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 4). Labeled bands at 155,000 and 97,000 daltons under non-reducing conditions (lanes 2 and 3 in Fig. 4) and 135,000 and 110,000 under reducing conditions (lanes 4 and 5 in Fig. 4), corresponding to GPIII and GPIII, respectively, were observed in the labeled GPIIb-III, preparation added to the tubes as well as in the material eluted from the TSP-coated tubes. These results indicate that our assay measures bound GPIIb-III.

Binding of TSP to Immobilized GPIIb-III—In 2 h, approximately 1.2 ng of TSP bound to immobilized GPIIb-III, calculated from the specific activity of the [125I]TSP added to the incubation mixture, whereas less than 0.4 ng bound im-
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**FIG. 2.** Concentration dependence of GPIII-III, binding to immobilized TSP. Solutions containing various amounts of $^{125}$I-labeled GPIII-III, were incubated with TSP-coated tubes for 2 h at room temperature. The tubes were then washed and counted, and the amount of protein bound was calculated from the specific activity of the $^{125}$I-GPIII-III, added. The data points are the mean of triplicate determinations, and the bars represent the standard deviation. The line drawn through the points is the best fit line obtained by regression analysis using the graphics program Sigmaplot (Jandel Scientific).

**FIG. 3.** Effect of Mg$^2+$ and Ca$^2+$ on the binding of GPIII-III, to immobilized TSP. TSP-coated tubes were incubated with 3 pg of $^{125}$I-GPIII-III, in binding buffer containing the various additions designated in the figure. The tubes were then washed and counted, and the amount of protein bound was calculated from the specific activity of the $^{125}$I-GPIII-III, added. The data points are the mean of triplicate determinations, and the bars represent the standard deviation.

As an additional control to rule out nonspecific interactions on the plastic surface of our solid-phase assay tubes, TSP as well as BSA were coupled to Sepharose, and the derivatized gels were examined for GPIII-III,-binding activity. As can be seen in Fig. 7, only TSP-Sepharose bound $^{125}$I-GPIII-III, which was eluted with NaCl. These results further support the conclusion that GPIII-III, specifically interacts with TSP.

**DISCUSSION**

It is clear from a number of recent studies that TSP plays a major role in cellular adhesion (15–17) and also functions
Anti-Fbg were incubated with binding of GPIIb-III. Unbound material was washed with binding buffer, and bound material eluted with binding buffer containing 0.25 M of purified immune or nonimmune IgG as designated in the figure. Anti-Fbg refers to anti-fibrinogen IgG; A2Ag and SSA6 are anti-GPIIb-III. mononuclear antibodies. The tubes were then washed and counted, and the amount of protein bound was calculated from the specific activity of the 125I-GPIIb-IIa added. The data points are the mean of triplicate determinations, and the bars represent the standard deviation.

FIG. 6. Effect of antibodies, fibrinogen, and peptides on the binding of GPIIb-IIa to immobilized TSP. TSP-coated tubes were incubated with 5 μg of 125I-GPIIb-IIa in binding buffer containing either 200 μg of fibrinogen, 100 μg of Gly-Arg-Glu-Ser-Pro (GRGESP), 100 μg of Gly-Arg-Gly-Glu-Ser-Pro (GRGDSGP), or 30 μg of purified immune or nonimmune IgG as designated in the figure. Anti-Fbg refers to anti-fibrinogen IgG; A2Ag and SSA6 are anti-GPIIb-IIa, III. monoclonal antibodies. The tubes were then washed and counted, and the amount of protein bound was calculated from the specific activity of the 125I-GPIIb-IIa added. The data points are the mean of triplicate determinations, and the bars represent the standard deviation.

FIG. 7. Interaction of GPIIb-IIa with TSP-Sepharose and BSA-Sepharose. 125I-GPIIb-IIa was dissolved in binding buffer and chromatographed on either a TSP-Sepharose or BSA-Sepharose column. Unbound material was washed with binding buffer, and bound material eluted with binding buffer containing 0.25 M NaCl. The elution profile (solid circles) was obtained from the TSP-Sepharose column and from BSA-Sepharose (open circles). The data points are the mean of triplicate determinations, and the bars represent the standard deviation. The inset shows the autoradiogram of the SDS-gel analysis of fractions 5, 6, and 7 obtained from the TSP-Sepharose column.

in platelet aggregation (18, 19). Of great interest is the identification of the cellular TSP receptor. Ash and co-workers (20) have presented evidence that an 88,000-dalton membrane glycoprotein present in platelets, endothelial cells, and a variety of human tumor cells is the membrane-binding site of TSP. This protein was identified as glycoprotein IV (GPIV) by the use of specific anti-GPIV antisera (21). Although TSP was shown to bind to monocytes and mediate platelet-monocyte adhesion, no functional role for GPIV in these adhesive interactions was described (22).

Our recent studies (7, 18) suggest that GPIIb-IIa, the cell surface heterodimeric glycoprotein complex that mediates platelet aggregation and other platelet adhesive functions by its interaction with fibrinogen, fibronectin, and von Willebrand factor (22), may mediate TSP-promoted platelet aggregation. We found that antibodies against GPIIb-IIa inhibited the TSP potentiation of thrombin-stimulated platelet aggregation (7, 18). That GPIIIa may function as a platelet TSP receptor is also suggested from studies showing co-localization of TSP and GPIIb-IIa on the surface of thrombin-stimulated platelets (9) and from a study showing that anti-GPIIb-IIa inhibits binding of TSP to activated platelets (6). Clearly, however, other TSP receptors must also exist on the platelet surface since thrombasthenic platelets that are deficient in GPIIb-IIa also bind TSP (23).

In order to determine whether GPIIb-IIa can serve as a platelet TSP receptor, a simple in vitro binding assay utilizing purified TSP and GPIIb-IIa was developed. Tubes were coated with either TSP or GPIIb-IIa, the corresponding radiolabeled GPIIb-IIa, or TSP was added, and the bound ligand was counted after extensive washing to remove unbound material. We found that either GPIIb-IIa bound to immobilized TSP, or TSP bound to immobilized GPIIb-IIa, in a time-independent manner after less than 2 h of incubation. The binding was saturable and could be inhibited by either antibodies against GPIIb-IIa, or TSP. Binding was dependent on the presence of the divalent cations Mg²⁺ and Ca²⁺, consistent with the results reported previously for the binding of TSP to activated platelets (24).

The stoichiometries in these solid-phase reactions do not reflect stoichiometries that might be expected in the fluid phase. In our study, approximately one molecule of GPIIb-IIa bound 100 molecules of TSP. A similar stoichiometry was observed by Leung and Nachman (25) for the interaction of fibrinogen with TSP absorbed to plastic microtiter plates. As pointed out by Leung and Nachman (25), the poor stoichiometry could be due to randomly oriented TSP on the plastic giving rise to significant steric hindrance of binding. Thus, in our system, only one out of every 100 absorbed TSP molecules is capable of interacting with fluid-phase GPIIb-IIa.

Our results provide direct evidence for the interaction of GPIIb-IIa with TSP. The monoclonal antibody specific to GPIIb-IIa, SSA6, inhibited binding of GPIIb-IIa to TSP by 60%, whereas anti-GPIIb-IIa (A3A6), specific for the heterodimeric complex, inhibited binding by 35%. These levels of inhibition were maximal in the presence of excess antibody. We interpret these results as suggesting that the TSP-binding epitope of GPIIb-IIa is complex and may be located closer to the GPIIa portion of GPIIb-IIa. We could also demonstrate the interaction of GPIIb-IIa with TSP by enzyme-linked immunosassay. In addition, our preparations of GPIIb-IIa bound fibrinogen to the same extent as TSP (data not shown). We could also demonstrate binding of GPIIb-IIa to TSP immobilized on Sepharose (Fig. 7), suggesting that our observed ligand binding was not due to an artificial modification of the receptor proteins from adsorption on the tube surface.

Previous efforts to demonstrate an interaction between TSP and GPIIb-IIa, have yielded conflicting results. Leung and Nachman (25), using an enzyme-linked immunosassay, could demonstrate an interaction between GPIIb-IIa, and fibrinogen but not between GPIIb-IIa, and TSP. Similarly, Pytel et al. (26), using GPIIb-IIa, incorporated into liposomes, could not demonstrate an interaction with TSP. However, in agreement with our present studies and our previous work showing that a GPIIb-IIa-like receptor in melanoma cells binds TSP (27), Lawler and co-workers (28, 29) recently showed that TSP immobilized on Sepharose bound GPIIb-IIa, ²H. I. Switalska and G. P. Tuszyński, unpublished observations.
as well as a related integrin receptor present on human endothelial cells, smooth muscle cells, monocyte-like cells, normal rat kidney cells, and normal and thrombasthenic platelets. However, in contrast to the findings of Lawler and co-workers (28, 29), we could not demonstrate inhibition of binding of GPIb-III to TSP in our solid-state assay by Arg-Gly-Asp-containing peptides (Fig. 6). The reason for these discrepancies is presently unknown, although differences in the biological activities of either purified TSP or GPIb-III, used in the various studies could account for some of the observed differences in binding. In conclusion, our studies suggest that GPIb-III may function as one of the receptors for TSP on the surface of activated platelets. During aggregation, TSP may not directly cross-link platelet aggregates but rather act synergistically with fibrinogen through GPIb-III on the platelet surface. TSP could either provide additional fibrinogen receptor sites on the platelet surface or increase the binding constant for fibrinogen by interaction with either fibrinogen or the GPIb-III complex. Finally, TSP could directly interact with GPIb-III and cross-link platelets through a fibrinogen-independent pathway of platelet aggregation, which has been suggested by two recent studies (30, 31). Further work is needed to differentiate between these possibilities.

REFERENCES
1. Lawler, J., Derick, L. H., Connolly, J. E., Chen, J.-H., and Chao, F. C. (1985) J. Biol. Chem. 260, 3762-3772
2. Tusznyski, G. P., Srivastava, S., Switalska, H. I., Holt, J. C., Cierniewski, C. S., and Niewiarowski, S. (1985) J. Biol. Chem. 260, 12240-12245
3. Lawler, J. W., Slattery, H. S., and Coligan, J. E. (1978) J. Biol. Chem. 253, 8609-8616
4. Galvin, N. J., Vance, P. M., Dixit, V. M., Fink, B., and Frazier, W. A. (1987) J. Cell Biol. 104, 1413-1422
5. Switalska, H. I., Niewiarowski, S., Tusznyski, G. P., Rucinski, B., Schmaier, A., and Morinelli, T. A. (1985) J. Lab. Clin. Med. 106, 690-700
6. Walf, R., Plow, E. F., and Ginsberg, M. H. (1986) J. Biol. Chem. 261, 6840-6846
7. Tusznyski, G. P., Rothman, V. L., Murphy, A., Siegler, K., and Knudsen, K. A. (1985) Blood 72, 109-115
8. Dixit, V. M., Haverstick, D. M., O'Rourke, K. M., Hennessey, S. W., Grant, G. A., Santoro, S. A., and Frazier, W. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3472-3476
9. Ash, A. S., Leung, L. K., Polley, M. J., and Nachman, R. L. (1985) Blood 66, 926-934
10. Bennett, J. S., Hoxie, J. A., Leitman, S. F., Vilaire, G., and Cines, D. B. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2417-2421
11. Bratt, L. F., Shattil, S. J., Kunicki, T. J., and Bennett, J. S. (1985) J. Biol. Chem. 260, 7875-7881
12. Knudsen, K. A., Smith, L., Smith, S., Karczewski, J., and Tusznyski, G. P. (1988) J. Cell Physiol. 136, 471-478
13. Laemmli, U. K. (1970) Nature 227, 680-685
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
15. Tusznyski, G. P., Rothman, V. L., Murphy, A., Siegler, K., Smith, L., Smith, S., Karczewski, J., and Knudsen, K. A. (1987) Science 236, 1570-1573
16. Roberts, D. B., Sherwood, J. A., and Ginsburg, V. (1987) J. Cell Biol. 104, 131-139
17. Varani, J., Dixit, V. M., Fligiel, S. E. G., McKeever, P. E., and Carey, T. E. (1986) Exp. Cell Res. 167, 376-390
18. Tusznyski, G. P., Rothman, V. L., Murphy, A., and Knudsen, K. (1987) Semin. Thromb. Hemostasis 13, 361-368
19. Leung, L. L. K. (1984) J. Clin. Invest. 74, 1764-1772
20. Ash, A. S., Barnwell, J., Silverstein, R. L., and Nachman, R. A. (1987) J. Clin. Invest. 79, 1054-1061
21. Tondon, N. N., Hines, A., and Jamieson, G. A. Blood 66, Suppl. 1, 1148 (abstr.)
22. Plow, E. F., Ginsberg, M. H., and Marguerie, G. A. in Biochemistry of Platelets (Phillips, D. R., and Shuman, M. A., eds) pp. 225-236, Academic press, Orlando, FL
23. Aiken, M. L., Ginsberg, M. H., and Plow, E. F. (1986) J. Clin. Invest. 78, 1713-1716
24. Aiken, M. L., Ginsberg, M. H., and Plow, E. F. (1987) Blood 69, 58-64
25. Leung, L. L. K., and Nachman, R. L. (1982) J. Clin. Invest. 70, 542-549
26. Pytel, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F., and Ruoslahti, E. (1985) Science 226, 1550-1552
27. Tusznyski, G. P., Karczewski, J., Smith, L., Murphy, A., Rothman, V. L., and Knudsen, K. A. (1989) Exp. Cell Res. 182, 473-481
28. Lawler, J., and Hynes, R. O. (1988) Blood 72, Suppl. 1, 329 (abstr.)
29. Lawler, J., Weinstein, R., and Hynes, R. O. (1988) J. Cell Biol. 107, 2351-2361
30. Newman, P. J., McEver, R. P., Does, M. P., Kunicki, T. J. (1987) Blood 69, 668-676
31. Cattaneo, M., Kinough-Rathbone, R. L., Lecchi, A., Bevilacqua, C., Packham, M. A., Mustard, J. F. (1987) Blood 70, 221-226