Isolation of the Thrombospondin Membrane Receptor

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Abstract

Thrombospondin (TSP), a 450-kD multifunctional glycoprotein with a broad tissue distribution, is secreted upon platelet stimulation, binds to the activated platelet surface, and supports platelet aggregation. We have identified and isolated an 88-kD membrane glycoprotein present in platelets, endothelial cells, monocytes, and a variety of human tumor cell lines that is the membrane binding site for TSP. Endogenous platelet TSP binding to thrombin- and ionophore-stimulated human platelets was inhibited in the presence of the monoclonal antibody OKM5. TSP binding to C32 melanoma cells and HT1080 fibrosarcoma cells was specific and also inhibitable with OKM5 Mab. Cell labeling followed by specific immunoprecipitation demonstrated biosynthesis of a single 88-kD glycoprotein. Binding of TSP to the isolated membrane protein was specific and saturable. These studies identify an 88-kD membrane glycoprotein that reacts with the monoclonal antibody, OKM5, and may function as the cellular TSP receptor.

Introduction

Adhesive macromolecules play a central role in biology. By mediating cell-cell and cell-substrate interactions, they provide a framework upon which cell migration and morphogenesis occur during development (1, 2). Adhesive phenomena are also necessary for normal hemostasis in the adult organism (3) and play a role in the metastatic behavior of certain tumors (4-13). Although some adhesive molecules are intrinsic to the cell membrane, most are ligands for cellular receptors (14-19).

Platelet aggregation is a useful paradigm for characterizing cell adhesion phenomena. In vivo endothelial cell damage or denudation results in platelet adhesion with subsequent degranulation and the recruitment of additional platelets to form a plug. Platelet alpha granules contain numerous adhesive proteins, including von Willebrand factor, fibronectin, fibrinogen, and thrombospondin (TSP),¹ that are secreted upon platelet stimulation, bind to the platelet membrane, and mediate aggregation (20-26). Fibrinogen links aggregating platelets by binding to the glycoprotein IIb/IIIa complex (GPIIb/IIIa) on opposing membranes (27). Von Willebrand factor and fibronectin share the

capacity to bind to GPIIb/IIIa and have in common a four amino acid cellular binding domain, Arg-Gly-Asp-Ser (28-36). The GPIIb/IIIa complex has recently been identified in a variety of cells other than platelets, including endothelial cells (37), smooth muscle cells, and fibroblasts (38). Thus, this receptor complex and family of adhesive proteins are likely to have broad biological importance. Thrombospondin, a major alpha granule glycoprotein (39-43), differs from this family of proteins in that binding to the platelet surface does not appear to be mediated by GPIIb/IIIa. Binding of TSP occurs normally on platelets from patients with Glanzmann's thrombasthenia—an inherited deficiency of platelet GPIIb/IIIa (44, 45). Thus, a separate receptor for TSP must exist.

TSP is an adhesive 450-kD multifunctional glycoprotein composed of three identical disulfide-linked chains (46-48). TSP has a broad tissue distribution and is synthesized by endothelial cells (49, 50), fibroblasts (51), smooth muscle cells (52), alveolar pneumocytes (53), monocytes (54), and neuroglial cells (55) and is incorporated into the matrix of growing cells (51, 56). TSP is known to bind heparin (39, 41), fibrinogen (57), fibronectin (58, 59), histidine-rich glycoprotein (60), collagen (61), and plasminogen (62). It has lectinlike properties (63) and is necessary for irreversible platelet aggregation (64). TSP has also recently been reported to mediate the adherence to endothelium of red cells parasitized with *Plasmodium falciparum* (65).

A monoclonal antibody (Mab), OKM5 (Ortho Diagnostic Systems Inc., Raritan, NJ), is reactive with platelets, endothelial cells, and monocytes (66, 67). It identifies an 88-kD glycoprotein, and has recently been found to inhibit the cytoadherence of parasitized red cells to endothelial cell monolayers and the human melanoma cell line C32 (68). These observations raise the possibility that the antigen identified by OKM5 Mab is the cellular TSP receptor.

We report the isolation of an 88-kD membrane glycoprotein reactive with OKM5 Mab that binds TSP and may function as the cellular receptor for TSP.

Methods

Materials. OKM5 monoclonal antibody was kindly provided by Dr. G. Goldstein (Ortho Diagnostics Systems Inc.). A10, a monoclonal antibody with reactivity against decay accelerating factor (69) was a gift from Dr. T. Kinoshita (New York University Medical Center). C6.7 a monoclonal antibody that inhibits platelet aggregation (70) was a gift from Dr. V. Dixit. Monoclonal anti-TSP antibodies 11.4 and 45.2 were prepared and characterized as reported (71). Thrombin was a gift from Dr. J. Fenton. A23187 was purchased from Calbiochem-Behring Corp., La Jolla, CA. ¹²⁵Iodine, ³H-mannose, ³H-fucose, ³H-galactosamine, and ³H-glucosamine were purchased from Amersham Corp., Arlington Heights, IL. TSP was prepared from human platelets and iodinated as previously described (60). Monospecific immunoaffinity-purified Fab antibody to TSP was prepared and iodinated as previously described (62). Rabbit polyclonal anti-GPIV IgG was a gift from Dr. G. Jamieson and Dr. N. Tandon (National Red Cross, Bethesda, MD). Monospecific rabbit polyclonal anti-GPV antisera (72) was a gift of Dr. K. J. Clemetson

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1. Abbreviations used in this paper: GP, glycoprotein; Mab, monoclonal antibody; MEM, minimum essential medium; TSP, thrombospondin.

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(Theodore Kocher Institute, Bern, Switzerland). The tetrapeptide Arg-Gly-Asp-Ser was purchased from Peninsula Laboratories, Belmont, CA.

Cells. C32 melanoma cells and HT1080 fibrosarcoma cells were obtained from American Type Culture Collection, Rockville, MD, and were maintained in culture in Eagle's minimum essential medium (MEM) and 10% fetal calf serum (FCS).

Cell binding studies. The expression of TSP on the surface of stimulated human platelets was measured with ^{125}I -anti TSP Fab. Platelet-rich plasma (0.5 ml) was incubated with ^{125}I -anti TSP Fab 4,000 cpm/ μg and 10 $\mu\text{g}/\text{ml}$ OKM5 Mab or a control anti-platelet monoclonal. Ionophore A23187 (10 $\mu\text{g}/\text{ml}$) was added for 2 min without stirring and the platelets were centrifuged for 8 min through silicone oil as previously described (64). Bound radioactivity was quantified with an Isodyne gamma counter (Searle, Amsterdam, Netherlands), and inducible TSP expression was calculated as the difference between counts obtained with resting and stimulated platelets. Thrombin-inducible TSP expression was measured similarly with gel-filtered human platelets. Thrombin (final concentration, 1 U/ml) was added to platelets incubated with ^{125}I -anti TSP Fab in the presence or absence of OKM5 Mab (final concentration, 10 $\mu\text{g}/\text{ml}$) without stirring for 1 min. Platelet pellets were obtained and counted as described above, and the difference between resting and stimulated bound radioactivity expressed as thrombin-inducible TSP.

The effect of OKM5 Mab (final concentration, 10 $\mu\text{g}/\text{ml}$) on ^{125}I -TSP binding to HT1080 cells was examined in suspension cultures. HT1080 cells grown in MEM with 10% FCS were centrifuged and resuspended in serum-free MEM at a concentration of 10^6 cells/ml. Triplicate 0.1-ml aliquots were incubated at 4°C with ^{125}I -TSP (16,000 cpm/pmol) over a range of concentrations. At 2 h, the cells were centrifuged through silicone oil as described previously, and bound radioactivity was counted. The effect of polyclonal rabbit anti-glycoprotein (GP) IV antibody on TSP binding was also examined at the 100 $\mu\text{g}/\text{ml}$ TSP point. Data were subjected to analysis with a modification of the Ligand program (73, 74).

The effect of OKM5 Mab (final concentration, 10 $\mu\text{g}/\text{ml}$) on ^{125}I -TSP binding to C32 melanoma cells was examined over a range of TSP concentrations at 4°C for 2 h. Cells grown in culture flasks were resuspended in Hepes buffer containing 0.02% EDTA and then resuspended in MEM for the binding studies that were performed in triplicate. At 2 h, the cells were centrifuged through silicone oil and the radioactivity of the pellets was counted. The time course of binding was examined similarly at 4°C at 200 $\mu\text{g}/\text{ml}$ TSP. Binding to monolayers grown in 24-well culture plates was performed similarly in MEM over a range of TSP concentrations at 4°C. At 2 h incubation, the wells were gently washed thrice with MEM and the cells were solubilized with 1% Triton X-100 in phosphate buffered saline (PBS), pH 7.4.

Isolation of the membrane binding site. Isolation of the 88-kD glycoprotein receptor from C32 melanoma cells and from human platelets was achieved with immunoaffinity column chromatography using an OKM5 Mab-Affigel 10 column. Cells were solubilized in PBS, pH 7.4, with 1% Triton X-100 in the presence of protease inhibitors. Following extensive washing, bound antigen was eluted from the column with 0.2 M glycine, pH 2.5, dialyzed against PBS, pH 7.4, and electrophoresed in a 7.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel (75). The material was electrophoretically transferred to nitrocellulose paper and incubated with purified TSP (50 $\mu\text{g}/\text{ml}$) in PBS, pH 7.4, with 0.05% Tween, 2 mM CaCl_2 , 2 mM MgCl_2 , and 1% bovine serum albumin. Following washing in several changes of buffer, the nitrocellulose was probed with colloidal gold-labeled goat anti-rabbit antibody and developed with Auroprobe (Janssen Life Sciences Products, Piscataway, NJ). Western blotting of the immunoisolated material was performed by incubating with rabbit anti-GPIV IgG (50 $\mu\text{g}/\text{ml}$), monospecific anti-GPV antisera (1:50 dilution), or normal rabbit antisera. Following extensive washing, detection was completed with peroxidase-conjugated goat anti-rabbit IgG and the colorimetric substrate 4-chloro-1-naphthol. Two-dimensional gel electrophoresis of the immunoisolated material was performed using a modification of the O'Farrell method as previously described (55).

Biosynthetic labeling of the membrane binding site. C32 cell cultures

grown in MEM with 10% FCS were labeled with ^3H -mannose, ^3H -fucose, ^3H -galactosamine, or ^3H -glucosamine for 24 h (0.5 mCi/ml) and solubilized in PBS, pH 7.4, with 1% Triton X. OKM5 Mab bound to Affigel 10 beads was used to immunoprecipitate antigen. Following extensive washing, the beads were boiled in 0.12 M Tris, pH 6.8, 2% SDS, 5% glycerol, 2.5% dithiothreitol, electrophoresed in a 7.5% SDS-PAGE gel, and autoradiography was performed.

TSP binding to the purified membrane binding site. The specificity of binding of TSP to the membrane protein was examined using purified material in enzyme-linked immunosorbent assay studies. TSP or albumin (4 $\mu\text{g}/\text{ml}$) were bound to the bottom of microtitre wells as previously described (62, 76). Purified OKM5 antigen (4 $\mu\text{g}/\text{ml}$) in PBS, Tween 0.05%, pH 7.4, was incubated in the wells for 90 min at 37°C. Wells were washed three times in buffer, and OKM5 Mab (2 $\mu\text{g}/\text{ml}$) was added to detect bound receptor. After additional washes, goat anti-mouse antibody conjugated to alkaline phosphatase was added and the bound OKM5 was quantified by the addition of the colorimetric substrate p-nitrophenyl phosphate.

The affinity of TSP for the isolated membrane protein was examined in binding studies using OKM5 antigen immobilized in microtitre wells. Immobilized OKM5 antigen was incubated with TSP or albumin in concentrations up to 400 $\mu\text{g}/\text{ml}$, and binding was quantified using alkaline phosphatase conjugated second antibody and colorimetric substrate. Binding was examined in the presence of anti-TSP monoclonals C6.7 (500 $\mu\text{g}/\text{ml}$), 11.4 (100 $\mu\text{g}/\text{ml}$), or 45.2 (100 $\mu\text{g}/\text{ml}$). Binding was also examined in the presence of the tetrapeptide Arg-Gly-Asp-Ser (0.12 mM and 1.2 mM) or 5 mM ethylene diamine tetra acetic acid. In an additional test of specificity, preincubation of the immobilized membrane binding site with OKM5 Mab (10 $\mu\text{g}/\text{ml}$) was done in control experiments before the addition of TSP.

Results

OKM5 Mab inhibits endogenous TSP binding to the platelet surface. Upon platelet stimulation, TSP is released from alpha granules and binds to the platelet surface (77). Recent studies have demonstrated TSP binding to resting as well as stimulated platelets (44). To examine the expression on the platelet surface of endogenous platelet TSP, we measured the binding of ^{125}I -labeled affinity-purified monospecific rabbit anti-TSP Fab to thrombin- and ionophore-stimulated platelets. Gel-filtered human platelets were stimulated with 1 U/ml thrombin for 2 min in the presence of ^{125}I -anti TSP Fab and with either OKM5 Mab (10 $\mu\text{g}/\text{ml}$) or a control anti-platelet Mab (Fig. 1). Thrombin-inducible TSP expression was completely inhibited in the presence of OKM5 Mab. The control anti-platelet Mab had no effect on TSP expression. Inhibition of TSP expression was not due to inhibition of release or aggregation, because in the presence of OKM5, thrombin aggregation was normal by aggregometry. In addition, platelet stimulation with the ionophore A23187 yielded similar results (Fig. 1). Ionophore-induced TSP expression was abrogated in the presence of OKM5 Mab.

OKM5 Mab inhibits TSP binding to human tumor cell lines. Using human fibrosarcoma HT1080 cells in suspension, binding of ^{125}I -TSP was studied over a range of concentrations, in the presence of 10 $\mu\text{g}/\text{ml}$ OKM5 Mab or irrelevant Mab. Data were subjected to analysis by the computerized Ligand program adapted for use with an IBM PC (EBDA and SCAFIT programs) and revealed an apparent dissociation constant of 200–500 nM and 80,000 sites per cell. HT1080 binding of ^{125}I -TSP was dramatically inhibited by 10 $\mu\text{g}/\text{ml}$ OKM5 Mab, but not control Mab. Binding was also inhibited by 50 $\mu\text{g}/\text{ml}$ polyclonal rabbit anti-GPIV antibody (Fig. 2).

The human melanoma cell line, C32, has been used for studies on the cytoadherence of *Plasmodium falciparum* par-

Figure 1. Inducible platelet TSP expression. Thrombin- and A23187-inducible TSP expression on the platelet surface as measured by ^{125}I -anti TSP Fab was studied. (A), Gel-filtered human platelets were activated with 1 U/ml thrombin in the presence of ^{125}I -anti TSP Fab. The platelets were pelleted through silicone oil and bound radioactivity was counted. Thrombin-inducible TSP expression in the presence of 10 $\mu\text{g}/\text{ml}$ OKM5 Mab was completely inhibited. (B), results of similar experiments using the calcium ionophore A23187 as the platelet agonist. Platelet-rich plasma was incubated with 10 μM A23187 for 2 min in the presence of ^{125}I -anti TSP Fab. Inducible TSP expression was completely inhibited by OKM5 Mab (10 $\mu\text{g}/\text{ml}$). A control anti-platelet monoclonal (A10) that reacts with decay accelerating factor (69) did not have any effect on TSP expression. Bars indicate $\pm$ SD.

asitized erythrocytes because of the relative ease of culture compared with human endothelial cells (78). OKM5 Mab reacts with C32 cells and has recently been reported to inhibit the cytoadherence of parasitized erythrocytes (68). We examined the binding of TSP to C32 cell suspensions and monolayers using ^{125}I -TSP in the presence of 10 $\mu\text{g}/\text{ml}$ of OKM5 Mab or a control Mab. At saturation, TSP binding was inhibited by 10 $\mu\text{g}/\text{ml}$ OKM5 (Fig. 3). Similar results were obtained with C32 cell monolayers. The time course of binding was examined at 4°C and a TSP concentration of 200 $\mu\text{g}/\text{ml}$ (Fig. 4). Binding was rapid with half maximal binding by 10 min and maximal binding at 90 min.

Figure 2. ^{125}I -TSP binding to HT1080 cells. HT1080 cells grown in suspension cultures were washed free of serum-containing medium and resuspended at a concentration of 10^6 cells/ml. ^{125}I -TSP was added at several concentrations, and incubation was carried out at 4°C for 2 h. Cells were pelleted through silicone oil and bound radioactivity was counted. Nonspecific binding as determined by the Ligand program (73, 74) was subtracted from total binding to determine specific binding. In the presence of 10 $\mu\text{g}/\text{ml}$ OKM5 Mab (open circles) specific ^{125}I -TSP binding was inhibited when compared with the binding in the presence of an irrelevant monoclonal (solid circles). Similar inhibition of TSP binding was observed with specific polyclonal anti-GPIV antibody (triangle). Error bars indicate $\pm$ SD.

Figure 3. ^{125}I -TSP binding to C32 melanoma cells. C32 cells grown in tissue culture flasks and resuspended were incubated with ^{125}I -TSP. Following incubation with TSP at 4°C for 2 h the cells were centrifuged through silicone oil and bound radioactivity was counted. Bar graph depicts binding at saturation (100 $\mu\text{g}/\text{ml}$). In the presence of OKM5 Mab, ^{125}I -TSP binding was dramatically inhibited. Error bars denote SD.

Isolation of the membrane TSP binding site. Using an OKM5 Mab affinity column, the binding site was isolated from C32 melanoma cells and platelets and electrophoresed in a 7.5% SDS-PAGE gel. The silver-stained gel is shown in Fig. 5. Lane 1 is the protein immunisolated from human platelets; lane 2 is derived from C32 melanoma cells. A single major band that migrates with an apparent molecular weight of 88 kD is present in both lanes. In two dimensional O'Farrell gels, the 88-kD material had an isoelectric point (pI) of 4.5–5.5 (not shown), consistent with previously published data on GPIV (79).

Biosynthetic labeling of the binding site. To demonstrate endogenous synthesis of the OKM5 antigen, immunoprecipitation with OKM5 Mab-sepharose was used to purify an 88-kD glycoprotein from ^3H -mannose, -fucose, -glucosamine, or -galactosamine-labeled C32 cells. Fig. 6 is an autoradiogram prepared from a 7.5% SDS-PAGE gel revealing a single band of 88 kD in lanes 1–4 (lane 1, mannose; lane 2, fucose; lane 3, glucosamine; lane 4, galactosamine). Lane 5 is a control lane of material immunoprecipitated by normal mouse IgG, no radioactive material is present.

TSP binding to the purified membrane protein. The immunisolated binding site was subjected to 7.5% SDS-PAGE, electrophoretically transferred to nitrocellulose paper and incubated with TSP (50 $\mu\text{g}/\text{ml}$) probed with affinity-purified monospecific anti-TSP Fab and visualized with a gold-labeled second antibody and the Auroprobe detection system (Janssen, Life Sciences Products). In Fig. 7, lane 1, an 88-kD platelet-derived species demonstrates TSP binding confirming the molecular weight of the TSP binding material. Similar findings were obtained with material immunisolated from C32 cells. The binding was specific, because control lanes with albumin or column flowthrough did not bind TSP. In addition, Western blotting of the immunisolated material using a specific polyclonal anti-GPIV an-

Figure 4. Time course of binding of ^{125}I -TSP to C32 melanoma cell monolayers. C32 cells grown in tissue culture flasks and then resuspended were incubated with ^{125}I -TSP (200 $\mu\text{g}/\text{ml}$) at 4°C. At various time points, the cells were pelleted through silicone oil, and the bound counts were quantified. Binding was rapid, with maximal

binding by 90 min and half-maximal binding by 10 min.

Figure 5. Immunoblot of the OKM5 antigen. Purified OKM5 antigen was obtained from C32 melanoma cells and from human platelets using an OKM5 immunoaffinity column. The purified material was subjected to 7.5% SDS-PAGE and silver stained. *Lane 1* is platelet derived; *lane 2* is derived from C32 melanoma cells. Both cells demonstrate the presence of a predominant 88-kD band.

Figure 6. Biosynthetic radiolabeling of OKM5 antigen. C32 melanoma cells were radiolabeled with ^3H -mannose, -fucose, -galactosamine, or -glucosamine (0.5 mCi/ml) for 24 h and OKM5 antigen was immunoprecipitated using OKM5 Mab coupled to Affigel 10 beads. Autoradiography reveals the presence of a single molecular weight species of 88 kD.

Figure 7. TSP binding to electrophoretic blot of the isolated OKM5 antigen; reactivity with anti-GPIV antibody. $\sim 10 \mu\text{g}$ of immunoisolated OKM5 antigen from platelets were run on a 7.5% SDS-PAGE gel, electrophoretically transferred to nitrocellulose paper, incubated with TSP (50 $\mu\text{g}/\text{ml}$) in PBS with 0.05% Tween, 2 mM CaCl_2 , 2 mM MgCl_2 , and 1% bovine serum albumin, and probed with a gold-labeled second antibody (*lane 1*). A single 88-kD band is present. *Lane 2* is a Western blot of OKM5 antigen with detection by polyclonal rabbit anti-GPIV antibody. A single specific band is present at 88 kD. Another band at 200 kD was present also in blots using normal rabbit IgG as a control and was not further identified.

tibody confirmed reactivity specifically with the 88-kD material (*lane 2*). A nonspecific band of apparent Mr of 200 kD was present in both experimental as well as control blots with normal rabbit antisera. Immunoblots using a specific anti-GPV antisera were negative; as were ligand blots using ^{125}I -labeled thrombin (data not shown).

To further examine the specificity of TSP binding to the OKM5 Mab antigen, several studies were performed with the purified material in ELISA binding experiments. TSP or albumin (4 $\mu\text{g}/\text{ml}$) were coated on microtitre wells and binding of the purified OKM5 antigen was quantified with OKM5 Mab followed by alkaline phosphatase conjugated goat anti-mouse IgG (Fig. 8). Binding of OKM5 antigen to immobilized TSP was more than 10-fold greater than that observed with immobilized albumin. These results show specific binding of the OKM5 antigen to TSP and exclude the possibility that the observed binding of TSP was due to a minor contaminant present in the preparation.

To define the characteristics of TSP binding to the purified receptor in an isolated system, microtitre wells with immobilized OKM5 antigen were incubated with TSP or albumin in concentrations up to 400 nM. Binding was quantified using alkaline phosphatase conjugated second antibodies followed by colorimetric substrate (Fig. 9). Preincubation of the immobilized antigen with OKM5 Mab in similar experiments reduced TSP binding by one half. The apparent Kd of the receptor-TSP binding in this system was ~ 50 nM. Albumin binding was not significant. Binding was inhibited in the presence of EDTA but

Figure 8. Binding of the isolated receptor to TSP. TSP or albumin (4 $\mu\text{g}/\text{ml}$) were coated on the bottom of plastic microtitre wells, and the binding of the purified OKM5 antigen (4 $\mu\text{g}/\text{ml}$) to the immobilized proteins was examined. Following incubation and washing, the wells were probed with OKM5 Mab followed by alkaline phosphatase-conjugated goat anti-mouse antibody, and O.D.₄₀₅ measured after the addition of the colorimetric substrate, p-nitrophenyl phosphate.

Figure 9. Affinity of the purified receptor for TSP. Purified OKM5 antigen (4 $\mu\text{g}/\text{ml}$) was coated on the bottom of plastic microtiter wells and the binding of purified platelet TSP (solid circles) or albumin (open circles) was studied over a range of concentrations. In this system saturable TSP binding occurred with an apparent K_d of ~ 50 nM. No significant binding of albumin to the receptor was observed.

was not inhibitable by the tetrapeptide Arg-Gly-Asp-Ser (Fig. 10).

To further examine the specificity of binding, TSP binding was examined in the presence of monoclonal anti-TSP antibodies. C6.7 (500 $\mu\text{g}/\text{ml}$), an anti-TSP monoclonal that inhibits the secretion-dependent phase of platelet aggregation (70) partially blocked TSP binding. Two anti-TSP monoclonals, that have no effect of platelet aggregation, 11.4 and 45.2 (100 $\mu\text{g}/\text{ml}$), completely blocked TSP binding to the immobilized receptor (data not shown). Thus, TSP binding to GPIV appears to be mediated by a domain or domains that are recognized by these antibodies.

Discussion

We have identified and isolated an 88-kD glycoprotein, present on platelets, endothelial cells, monocytes, and a variety of human tumor cell lines, that functions as the membrane TSP binding site. The membrane protein is reactive with OKM5 Mab as well as polyclonal anti-GPIV antibody. In binding studies using whole cells, OKM5 Mab as well as polyclonal anti-GPIV antibody inhibited TSP binding, and in studies on human platelets OKM5 Mab inhibited activation inducible endogenous TSP expression. Biosynthetic labeling with ^3H -mannose, -fucose, -galactosamine, or -glucosamine demonstrated synthesis of the

88-kD glycoprotein. The isolated protein bound TSP in a specific and saturable manner with an apparent K_d on plastic coated surfaces of 50 nM.

Recently, OKM5 Mab has been reported to identify an antigen on platelets that reacts with antisera to a 90-kD major surface glycoprotein referred to as GPIV (80) or GPIIb by other nomenclature (79). The material we immunoisolated with OKM5 Mab likewise reacts with anti-GPIV antibody, and TSP binding to cells was inhibited by the polyclonal antibody, thus supporting the conclusion that GPIV functions on the platelet surface and the two cell lines studied, as the membrane TSP binding site. GPIV is increased in platelets from patients with myeloproliferative syndromes, and it is interesting to note that yields of TSP receptor from platelets of such patients were several-fold higher than yields from normal donor platelets (A. Asch, personal observation). Another platelet membrane glycoprotein of similar molecular weight is GPV. GPV migrates with a molecular weight of 82 kD and has a pI of 7–8 (72). GPV binds thrombin and is itself a thrombin substrate—being cleaved by thrombin to 70 kD (72). To exclude the possibility that the receptor we isolated was GPV we examined the behavior of the immunoisolated material in two dimensional O'Farrel gels. The 88 kD material we isolated migrated with a pI range of 4.5–5.5. In Western blots, the material was nonreactive with monospecific anti-GPV antisera. Thrombin did not bind to the immunoiso-

Figure 10. Effect of EDTA and the tetrapeptide Arg-Gly-Asp-Ser (RGDS) on TSP binding to the purified receptor. Purified OKM5 antigen (4 $\mu\text{g}/\text{ml}$) was coated on the bottom of plastic microtiter wells, and the binding of purified TSP (50 $\mu\text{g}/\text{ml}$) was examined in the presence of 2 mM CaCl_2 with or without RGDS at 0.12 mM and 1.2 mM, or 5 mM EDTA. Bars depict the means of quadruplicate points. In the presence of 5 mM EDTA TSP binding was abolished. No effect on binding was observed with RGDS.

lated receptor in ELISA studies or ligand blots, and the immunoisolated material was not a thrombin substrate. The TSP-binding membrane protein we have isolated is most likely GPIV.

TSP is secreted upon platelet activation and binds to the platelet surface. Our data demonstrate that this binding is mediated by a specific 88-kD membrane receptor previously referred to as GPIV. ¹²⁵I-TSP binding to resting and stimulated platelets has recently been examined and two classes of TSP binding, inducible and noninducible, have been found (81). The possibility exists that conformational changes occurring in a single receptor upon activation may mediate both types of TSP binding. TSP binding to thrombasthenic platelets is normal under resting as well as stimulated conditions (44, 45) consistent with the existence of a TSP membrane receptor other than GPIIb/IIIa. In our studies, inducible TSP expression on the platelet surface was completely abrogated by the OKM5 Mab. The effect was not due to inhibition of thrombin activation, because the same results were obtained with ionophore stimulation and platelet aggregation revealed complete aggregation under the conditions we used.

The demonstration of specific and saturable TSP binding to C32 melanoma cells and to the fibrosarcoma cell line HT1080 raises the possibility that TSP localization on the cell surface may be an important component in the malignant phenotype of these cells. The mechanism by which malignant cells give rise to a gross metastasis has been described as a multistep process requiring detachment of a viable cell from the primary tumor mass into the circulation, survival within the circulation, adhesion to and migration through the endothelium and subendothelium of the target tissue, and successful growth in the new environment (82). The factors that mediate these steps are not well known but several intriguing possibilities exist. Some data have been provided that implicate platelet-tumor cell interactions in the process of tumor cell metastasis, and in some murine models, antiplatelet agents inhibit the development of metastatic lesions (83). The platelet-TSP-tumor cell aggregate may serve as a vehicle for efficient transport through the circulation. Alternatively, endogenous TSP production by tumor cell lines such as HT1080 (A. Asch, personal observation) may lead to binding of the adhesive macromolecule to GPIV on the platelet surface and serve as a site for further platelet-tumor cell interaction and substrate adhesion. We have previously demonstrated that TSP can bind plasminogen and modulate plasmin generation at a surface by tissue plasminogen activator (62). Thus, TSP may support egress of malignant cells through basement membrane by localizing plasmin generation at the cell surface, or promote tumor cell adhesion by virtue of its lectinlike activity. TSP synthesis in smooth muscle cells and glial cells is regulated by platelet-derived growth factor (84). Likewise, the TSP receptor, GPIV, may also be modulated by the cell cycle or growth factors.

The discovery of a shared cellular attachment sequence in fibrinogen, fibronectin, vitronectin, von Willebrand factor (28-36, 44, 45, 85), and the implied existence of a family of receptors (3) for these molecules suggests that they have broad biological importance. TSP also has a broad tissue distribution but differs from the other adhesion proteins in that it does not bind to GP IIB/IIIa (57) or like molecules (86). Binding of TSP to the platelet surface has recently been shown to be independent of GPIIb/IIIa (44). We have identified the membrane-binding site for TSP that may function as a cell receptor for this large ubiquitous cellular and matrix glycoprotein. Whether this membrane-binding site leads to cellular activation via a second messenger

or functions solely as an adhesive protein receptor remains to be determined. Whereas the binding of TSP to this membrane protein is calcium dependent, it does not appear mediated by an RGD sequence that has been identified in the cloned TSP message (87). This raises the possibility that GPIV may belong to a class of adhesive membrane receptors that support cell adhesion in an RGD-independent manner. The distribution of the receptor in platelets, endothelial cells, monocytes, and tumor cells parallels TSP's broad distribution and provides a mechanism by which TSP may function as a modulator of membrane function.

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