Characterization of the Platelet Agglutinating Activity of Thrombospondin†

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ABSTRACT: Thrombospondin (TSP) is a glycoprotein secreted from the α-granules of platelets upon activation. In the presence of divalent cations, the secreted protein binds to the surface of the activated platelets and is responsible for the endogenous lectin-like activity associated with activated platelets. Platelets fixed with formaldehyde following activation by thrombin are agglutinated by exogenously added TSP. Fixed, non-activated platelets are not agglutinated. The platelet agglutinating activity of TSP is optimally expressed in the presence of 2 mM each of Mg^{2+} and Ca^{2+}. Reduction of the disulfide bonds within the TSP molecule inhibits its platelet agglutinating activity. TSP bound to the surface of fixed, activated platelets can be eluted by the addition of disodium ethylenediaminetetraacetate. This approach was exploited to identify the region of the TSP molecule containing the platelet binding site. The binding site resides within a thermolytic fragment of TSP with M_r 140 000 but is not present in the M_r 120 000 fragment derived from the polypeptide of M_r 140 000. Since both the M_r 140 000 and 120 000 fragments contain fibrinogen binding sites, this finding suggests that the binding of TSP to the platelet surface requires interaction with other platelet surface components in addition to fibrinogen. The observation that fibrinogen only partially inhibits the TSP-mediated agglutination of fixed, activated platelets is consistent with this interpretation.

Thrombospondin (TSP),[1] also referred to as thrombin-sensitive protein (Baenziger et al., 1971, 1972) and glycoprotein G (Phillips et al., 1980), is a high molecular weight platelet glycoprotein which is secreted from α-granules upon platelet activation (Gartner et al., 1981a). The trimeric protein is composed of apparently identical subunits of M_r 180 000 [as determined by SDS–PAGE in the Laemmli (1970) system] linked by disulfide bonds (Baenziger et al., 1972; Lawler et al., 1978; Margossian et al., 1981; Dixit et al., 1984b). Sedimentation equilibrium studies yield a somewhat lower value of subunit molecular weight, 140 000 (Margossian et al., 1981). TSP is composed of several relatively protease-resistant domains containing binding sites for a variety of macromolecules such as heparin (Lawler et al., 1978; Lawler & Slayter, 1981; Dixit et al., 1984b), fibrinogen (Leung & Nachman, 1982; Dixit et al., 1984a), fibronectin (Lahav et al., 1982), collagen (Lahav et al., 1982; Mumby et al., 1984), and plasma histidine-rich glycoprotein (Leung et al., 1984). Recently, the high-affinity heparin binding domain has been localized to the amino terminus of the TSP polypeptide (Coligan & Slayter, 1984; Dixit et al., 1984b). TSP has been identified as the endogenous platelet lectin which is expressed on the platelet surface following platelet activation and which mediates the hemagglutinating activity of activated platelets (Gartner et al., 1977; Jaffe et al., 1982). The hemagglutinating activity of purified TSP has recently been characterized in detail and a proteolytic fragment of the TSP molecule possessing the red cell binding region identified (Haverstick et al., 1984).

Physical studies of TSP indicate that the protein is dependent upon Ca^{2+} for retention of its native structure (Lawler et al., 1982; Lawler & Simons, 1983). Limited proteolytic degradation of the protein in the presence or absence of Ca^{2+} produces strikingly different polypeptide fragments (Lawler & Slayter, 1981; Lawler & Simons, 1983). In addition, the sedimentation coefficient of the protein is different in the presence of EDTA or Ca^{2+}, as is the intrinsic viscosity, suggesting an unfolding of the protein in the absence of Ca^{2+}. This conclusion is supported by electron microscope images obtained after low-angle rotary shadowing (Margossian et al., 1981).

Although originally described as a platelet glycoprotein, TSP has since been shown to be synthesized and secreted by endothelial cells (McPherson et al., 1981; Mosher et al., 1982; Raugi et al., 1982), fibroblasts (Raugi et al., 1982; Jaffe et al., 1983), smooth muscle cells (Raugi et al., 1982), and type II pneumocytes (Sage et al., 1983) when these cell types are grown in culture. In addition, as mentioned above, TSP has been shown to bind to several extracellular matrix macromolecules. McKeown-Longo et al. (1984) have observed that heparin can inhibit the binding of TSP to fibroblasts and their matrices in culture. Collectively, these observations suggest that TSP may play a broad role in cell–cell and cell–substrate interactions or in the organization of the extracellular matrix. These postulates have yet to be supported by definitive evidence.

Over the last several years, considerable progress has been made in understanding the nature of the interactions involved in platelet aggregation. These studies have been the subject of a recent review (Berndt & Phillips, 1981). To summarize, the interaction of an agent such as thrombin or ADP with its receptor results, in the presence of divalent cations, in the development of fibrinogen binding activity by the platelet membrane glycoprotein IIb/III complex. Platelet mem-

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[1] Abbreviations: TSP, thrombospondin; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; M_r, apparent molecular weight; EDTA, disodium ethylenediaminetetraacetate; TBS, Tris-buffered saline (0.15 M sodium chloride and 0.05 M Tris, pH 7.4); ACD, citric acid (8 g/L), trisodium citrate (22 g/L), and glucose (24.5 g/L), pH 4.5; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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brane-bound fibrinogen appears to play a major role in bridging adjacent platelets. TSP can bind to this fibrinogen on the platelet surface (Leung & Nachman, 1983) and may serve to augment platelet aggregation since inhibitors of the lectin-like activity of TSP inhibit thrombin-induced platelet aggregation (Gartner et al., 1978; Agam et al., 1984). Studies with platelets from afibrinogenemic individuals have suggested that platelet surface-bound fibrinogen serves as the platelet receptor for TSP (Gartner et al., 1981b). Altered forms of TSP have been described in patients with essential thrombocythemia and abnormal platelet aggregation (Booth et al., 1984).

In the investigation described herein, we have employed platelets fixed before or after activation with thrombin to characterize the platelet agglutinating activity of purified, Ca^{2+}-replete TSP. We have also identified a region of the TSP molecule which retains the ability to bind to the platelet surface.

Materials and Methods

Materials. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Human thrombin was a generous gift from Dr. Joseph Miletich, Washington University School of Medicine, and heparan sulfate was from Dr. Alfred Linker, University of Utah. Fibrinogen (grade L) was from Kabi (Stockholm, Sweden). Beef lung heparin was obtained from Upjohn (Kalamazoo, MI) and porcine mucosal heparin from Sigma. Compounds for use in SDS–PAGE were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA). Iodobeads were obtained from Pierce Chemical Co. (Rockford, IL), and carrier-free $Na^{125}I$ was purchased from New England Nuclear (Boston, MA).

Platelet Preparation. Blood was drawn from healthy volunteers and anticoagulated with $^1/_{10}$th volume of ACD. Platelet-rich plasma was prepared by centrifugation at 180*g* for 15 min at room temperature. The platelet-rich plasma was then centrifuged at 1100*g* for 10 min to sediment the platelets. The platelets were suspended in 0.14 M NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 0.55 mM glucose, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.4, and $^1/_{10}$th volume of ACD. Following a second centrifugation at 1100*g* for 10 min, the platelets (5×10^8/mL) were suspended in 0.05 M Tris, pH 7.4, and 0.15 M NaCl (TBS). The platelets were activated with 1 unit/mL human thrombin, and the reaction was stopped by the addition of 2 units/mL hirudin after 15 min at room temperature. The suspension was fixed with the addition of an equal volume of 3% formaldehyde in TBS and placed at 4 °C for 2–18 h. No differences in the behavior of platelets fixed for 2 or 18 h were observed. The fixed, activated platelets were then washed 3 times in TBS and adjusted to a final count of (4–5) $\times 10^8$/mL prior to use. Nonactivated fixed platelets were prepared in the same manner except that the thrombin and hirudin were omitted.

TSP Preparation. The procedure for purification of Ca^{2+}-replete TSP from platelet concentrates has recently been described in detail (Dixit et al., 1984a; Haverstick et al., 1984). Briefly, washed platelets were activated by the addition of human thrombin (2 units/mL), and the reaction was stopped with hirudin (4 units/mL) and PMSF (2 mM). Platelet aggregates and fibrillar material were removed by centrifugation. The supernatant was passed over a gelatin–Sepharose column equilibrated in 0.02 M Tris (pH 7.6), 0.15 M NaCl, and 1 mM $CaCl_2$ (buffer A) at 4 °C to remove any contaminating platelet fibronectin followed by chromatography on a heparin–Sepharose column equilibrated in buffer A. TSP was eluted from the heparin–Sepharose column by increasing the concentration of NaCl to 0.6 M. The preparation was then passed over a Bio-Gel A5M column to remove any remaining low molecular weight contaminants and to equilibrate the protein in 0.02 M Tris, pH 7.4, 0.15 M NaCl, and 1 mM $CaCl_2$ (buffer B). The protein was stored in buffer B containing 20% (w/v) sucrose at −70 °C prior to use.

TSP was radiolabeled with ^{125}I in a 5-min labeling procedure employing Iodobeads. Radiolabeled protein was separated from free iodide by gel filtration on a Sephadex G-10 column. The ^{125}I-labeled TSP was electrophoretically homogeneous and possessed agglutinating activities identical with those of the native protein.

Aggregation Assay. The platelet agglutinating activity of TSP was determined in essentially the same manner as the hemagglutinating activity of TSP (Haverstick et al., 1984). TSP was serially diluted in 25 μL in TBS in the wells of a microtiter "V" plate (Dynatech Laboratories, Alexandria, VA). Since preliminary experiments showed no effect of diluted sucrose solutions on the aggregation assay, TSP preparations were not routinely dialyzed prior to use. Salts, proteoglycans, protein fragments, etc. were added in 25-μL aliquots to each well. The reaction was initiated by the addition of 50 μL of the fixed platelets and the microtiter plate shaken on a Hyperion (Miami, FL) micromix shaker for 1 h at room temperature. The plate was then read against a black background with indirect lighting. The results are presented as the minimum concentration of TSP required to produce detectable platelet agglutination. Positive wells contained visible aggregates, whereas negative wells appeared cloudy due to the uniform suspension of platelets maintained by shaking during the course of the assay (see Figure 1). Unless otherwise indicated, all experiments were conducted in the presence of both 2 mM $CaCl_2$ and 2 mM $MgCl_2$.

Proteolytic Fragmentation of TSP. Partial proteolysis of TSP was carried out with thermolysin (bacterial protease type X, Sigma Chemical Co.) at an enzyme:substrate ratio of 1:100 (w/w). The digestion was allowed to proceed for 1 h at room temperature and was stopped by the addition of 3 μg of phosphoramidone per microgram of thermolysin. The digest was used without further manipulation.

SDS–PAGE. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in a slab gel apparatus using the discontinuous gel system described by Laemmli (1970). Protein was identified by the silver stain method using the system described by Merrill et al. (1981).

Protein Determination. Protein was quantitated by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

Results

Specificity of TSP-Induced Aggregation for Activated Fixed Platelets. Expression of the endogenous lectin-like activity of platelets is dependent upon activation of the platelets by an agent such as thrombin (Gartner et al., 1977). TSP secreted by activated platelets becomes bound to the platelet surface in the presence of divalent cations (Baenziger et al., 1971, 1972). We, therefore, sought to ascertain that agglutination of fixed platelets by exogenously added TSP was only possible when the platelets were activated prior to fixation. Platelets were formaldehyde fixed both before and after thrombin activation (see Materials and Methods) and used in the aggregation assay with exogenously added TSP in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$ (see cation requirements below). Figure 1 shows the results of such an experiment. Row A contains fixed, activated platelets and

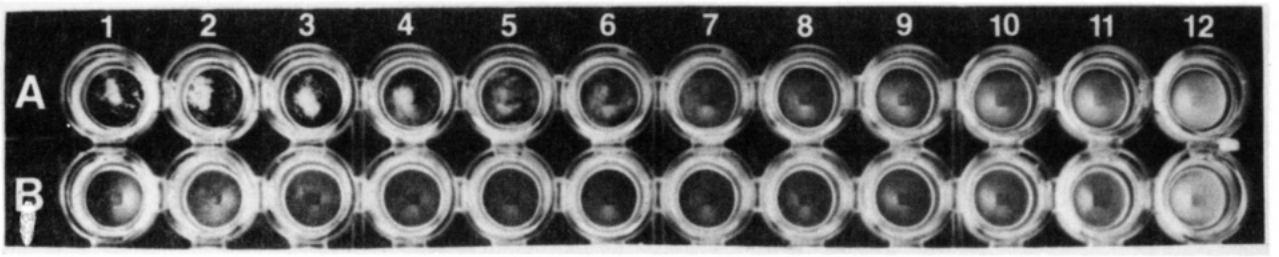

FIGURE 1: Aggregation of fixed platelets in the presence of exogenous TSP. Platelets from healthy volunteers were washed and fixed with formaldehyde either with or without prior activation with thrombin according to the procedure outlined under Materials and Methods. Isolated TSP in buffer B was serially diluted in TBS in 11 wells of each row of the microtiter plate. The final concentration in well 1 was 100 μg/mL. Well 12 served as a negative control (no exogenous TSP). Twenty-five microliters of TBS plus 8 mM $MgCl_2$ and 8 mM $CaCl_2$ was added to each well, and the assay was initiated with the addition of 50 μL of fixed, activated platelets (row A) or fixed, nonactivated platelets (row B) in TBS. The plate was shaken at room temperature and read after 1 h.

Table I: Divalent Cation Requirements for TSP-Induced Aggregation of Fixed, Activated Platelets

conditions	min eff TSP concn (μg/mL)[a]	conditions	min eff TSP concn (μg/mL)[a]
TBS	>88	TBS	
+5 mM EDTA	>88	+2 mM $MgCl_2$ +	3
+2 mM $MgCl_2$	>88	2 mM $CaCl_2$	
+2 mM $CaCl_2$	44	+4 mM $MgCl_2$	>88
+1 mM $MgCl_2$ +	11	+4 mM $CaCl_2$	44
1 mM $CaCl_2$		+4 mM $MgCl_2$ +	3
		4 mM $CaCl_2$	

[a] Minimum effective concentration of TSP causing aggregation.

serially diluted TSP. The last well containing agglutinated platelets is number 6. The TSP concentration in this well was 3 μg/mL. When fixed, activated platelets were employed, the minimal concentration of TSP required to produce agglutination was consistently observed to be 1–3 μg/mL. Row B contains fixed, nonactivated platelets. There are no positive wells. Fixed, activated platelets were used in all subsequent experiments unless otherwise indicated.

Divalent Cation Requirements for Platelet Agglutinating Activity. The hemagglutinating activity of TSP has recently been shown to be optimally expressed in the presence of 2 mM Ca^{2+} and 2 mM Mg^{2+} (Haverstick et al., 1984). The divalent cation requirements for the platelet agglutinating activity of purified TSP are shown in Table I. Mg^{2+} alone did not effectively support the platelet agglutinating activity of TSP. Ca^{2+} alone only weakly supported the platelet agglutinating activity of TSP. In the presence of both Ca^{2+} and Mg^{2+}, however, the concentration of TSP required to agglutinate fixed, activated platelets was greatly reduced. The presence of both Ca^{2+} and Mg^{2+}, each at a final concentration of 2 mM, provided optimal activity. No increase in platelet agglutinating activity was observed with further increases in the divalent cation concentrations. On the basis of these results, all subsequent experiments were conducted in the presence of TBS containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$.

Effect of Sulfhydryl Reduction on Aggregation Activity. Thrombospondin is composed of three apparently identical subunits of M_r 180000 linked by disulfide bonds (Baenziger et al., 1972; Lawler et al., 1978; Margossian et al., 1981; Dixit et al., 1984b). The effect of reduction of TSP disulfide bonds on the platelet agglutinating activity of TSP was examined by incubating TSP with increasing concentrations of DTT for 1 h at 37 °C and then testing the reduced protein in the aggregation assay. Figure 2 shows the effect of reduction on TSP structure as revealed by SDS–PAGE and on the platelet agglutinating activity. At DTT concentrations of 5–10 mM, some of the interpolypeptide chain disulfide bonds but not all

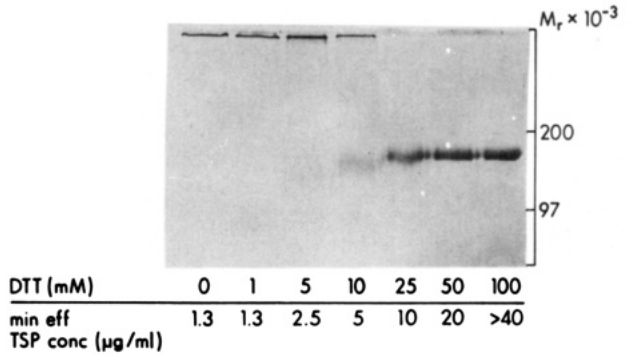

FIGURE 2: Effect of disulfide bond reduction on TSP structure and aggregation activity. Isolated TSP (500 μg/mL) was incubated with dithiothreitol (DTT) at various concentrations for 1 h at 37 °C. An aliquot of each sample was diluted with SDS sample buffer in the absence of any additional reducing agent and applied to a 6% acrylamide gel. Protein bands were identified by the silver stain method (Merrill et al., 1981). An additional aliquot of each sample was serially diluted in the aggregation assay. The minimal concentration of the reduced TSP required to produce aggregation of fixed, activated platelets is indicated below the corresponding lane of the gel.

of the intrachain disulfides have been reduced, producing a faint, broad band of somewhat lower molecular weight than the fully reduced chains which are produced at 25 mM DTT. Although the protein appeared to be totally reduced to its M_r 180000 subunit in the presence of 25 mM DTT, significant aggregation of the fixed, activated platelets was still observed. The platelet agglutinating activity was further reduced by incubation with higher concentrations of DTT. The fixed, activated platelets did not aggregate in the presence of the reducing agent unless exogenous TSP was added (not shown). These results differ from the observed effect of disulfide bond reduction on the hemagglutination activity of TSP, where disulfide bond reduction and loss of agglutination activity were more closely correlated (Haverstick et al., 1984). These results suggest that the ability of TSP to bind to the platelet surface might be dependent upon the integrity of some intrapolypeptide chain disulfide bonds which are more resistant to reduction than the interpolypeptide chain disulfide bonds. It seems likely as well that the TSP polypeptide chains remain associated, at least to some extent, after reduction of the interchain disulfides under nondenaturing conditions in order to retain platelet agglutinating activity. It is, however, also possible that additional reduction of interchain disulfide bonds occurs after the additional protein unfolding induced by the introduction of SDS, thus accounting for the apparent discrepancy, at least in part. It is clear, nevertheless, that intact disulfide bonds are required for optimal expression of the platelet agglutinating activity of TSP.

Table II: Inhibition of TSP-Induced Aggregation of Fixed, Activated Platelets

conditions	concn	min eff TSP concn[a] (μg/mL)
	(A) Effect of Fibrinogen	
control		2
fibrinogen	0.25 mg/mL	13
	1.25 mg/mL	13
	(B) Effect of Heparin	
control		2
heparin	0.4 unit/mL	3
	0.8 unit/mL	3
	1.6 units/mL	6
	3 units/mL	6
	6 units/mL	13
	12.5 units/mL	13
	25 units/mL	13
	(C) Effect of Proteoglycans	
control		2
heparin	74 μg/mL (12.5 units/mL)	13
heparan sulfate	2.5 mg/mL	6
chondroitin sulfate	2.5 mg/mL	2
dermatan sulfate	1.25 mg/mL	2
hyaluronic acid	0.6 mg/mL	6
keratan sulfate	0.25 mg/mL	2

[a] Minimum effective concentration of TSP causing aggregation.

Inhibition of TSP-Induced Agglutination of Fixed, Activated Platelets by Fibrinogen and Heparin. Like the cell-surface and extracellular matrix protein fibronectin (Hynes & Yamada, 1982), thrombospondin has been shown to be composed of discrete, relatively protease-resistant domains with binding sites for a number of other macromolecules. Binding sites for fibrinogen and heparin have been characterized in our laboratories (Dixit et al., 1984a,b). In addition, it has been suggested that fibrinogen, bound to the glycoprotein IIb/III complex on the membrane following platelet activation, serves as the binding site for TSP (Gartner et al., 1981b). We have, therefore, examined the effects of fibrinogen and heparin on the TSP-induced agglutination of fixed, activated platelets. Fibrinogen, when added to the assay system, was found to be an effective inhibitor of the aggregation activity. The presence of 250 μg/mL fibrinogen produced an 8-fold increase in the amount of TSP required to produce detectable platelet agglutination (Table IIA). The addition of higher concentrations of fibrinogen, however, did not further inhibit the platelet agglutinating activity of TSP.

Heparin, which was shown to be a potent inhibitor of the hemagglutinating activity of TSP (Haverstick et al., 1984), was also examined in the platelet agglutination assay (Table IIB). Although the compound was inhibitory to the same extent as fibrinogen (a maximal 8-fold reduction in activity was observed), the concentrations required were higher than those needed for inhibition in the hemagglutination assay (Haverstick et al., 1984). Both bovine lung heparin and porcine mucosal heparin were tested, and no differences in inhibitory activity were observed. To demonstrate that the heparin inhibition was not due solely to the presence of a highly charged molecule in the assay system, other proteoglycans were also examined. As shown in Table IIC, heparin was the most effective inhibitor, although both heparan sulfate and hyaluronic acid did cause a slight reduction in the platelet agglutinating activity of exogenous TSP when present in high concentrations.

Binding and Elution of TSP from Platelets. The divalent cation dependence of the hemagglutinating activity of TSP was recently exploited to identify proteolytic fragments of TSP containing the red cell binding site (Haverstick et al., 1984). A similar approach was used to study the interaction of TSP with platelets. Fixed, activated or fixed, nonactivated platelets were incubated with purified TSP in TBS containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ for 30 min at room temperature. The platelets were then pelleted by centrifugation and washed once with the divalent cation containing buffer, and any TSP bound in a divalent cation dependent manner was then eluted by the addition of TBS containing 5 mM EDTA. Eluted material was identified by SDS–PAGE. The results of such an experiment are shown in Figure 3. When fixed, activated platelets were employed, TSP was bound to the platelets and subsequently eluted by the addition of EDTA. When the experiment was performed with fixed, nonactivated platelets, no detectable TSP was recovered in the EDTA-eluted fraction. Thus, divalent cation dependent binding of TSP appears to occur to a significant degree to the surface of activated but not nonactivated platelets. This conclusion is consistent with the observation described above (Figure 1) that platelets must be activated before they can be agglutinated by exogenously added TSP. Obviously, a very small degree of binding below the limit detectable by silver staining would not be detected by this procedure.

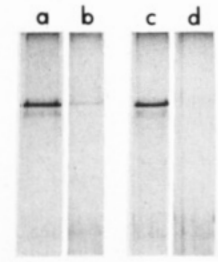

FIGURE 3: Binding of TSP to activated and nonactivated formaldehyde-fixed platelets. 150 μg of TSP in TBS plus 2 mM $MgCl_2$ and 2 mM $CaCl_2$ was incubated with 5×10^8 platelets fixed with formaldehyde either with (lanes a and b) or without (lanes c and d) prior activation with thrombin in a final volume of 1 mL. After a single wash of 3 mL, bound material was eluted with 400 μL of TBS containing 5 mM EDTA. Samples were prepared in 5% 2-mercaptoethanol and applied to a 7.5% acrylamide gel. Protein was identified by the silver stain method. (a) Unbound TSP; (b) material eluted from fixed, activated platelets; (c) unbound TSP; (d) material eluted from fixed, nonactivated platelets.

In experiments employing ^{125}I-labeled TSP, which possesses agglutinating activity identical with that of the native molecule, under conditions identical with those described above, 0.9% of the added TSP could be eluted with EDTA from the surface of fixed, activated platelets whereas only 0.01% of the added radioactivity could be recovered from the surface of fixed, nonactivated platelets upon the addition of EDTA. The material recovered upon the addition of EDTA represented 60–70% of the radioactivity associated with fixed, activated platelets.

Identification of TSP Fragments with Platelet Binding Activity. The approach outlined above was extended to the identification of proteolytic fragments of TSP which retain platelet binding activity. When TSP is subjected to limited digestion with thermolysin (enzyme:substrate ratio of 1:100 for 60 min at 25 °C), three major fragments are produced with molecular weights of 140 000, 120 000, and 25 000 (Dixit et al., 1984a,b; Haverstick et al., 1984). Smaller quantities of as yet uncharacterized fragments are also present (Figure 4). A precursor–product relationship appears to exist between the M_r 140 000 and 120 000 fragments, and both contain a binding site for fibrinogen (Dixit et al., 1984a). Only the M_r 140 000 fragment, however, was found to retain a red cell binding site (Haverstick et al., 1984). The M_r 25 000 fragment is derived

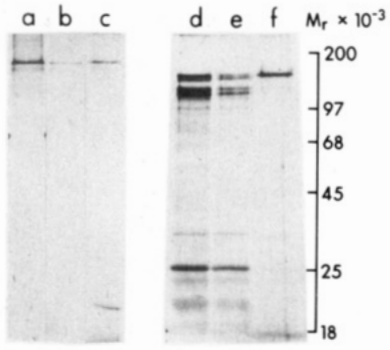

FIGURE 4: Identification of the platelet binding domain of TSP. Fixed, activated platelets (5×10^8) were incubated with 200 μg of intact TSP or thermolysin-digested TSP for 1 h at room temperature in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$ in a volume of 1 mL. The cells were centrifuged and washed 1 time with 3 mL of the incubation buffer. Bound material was then eluted with 400 μL of TBS containing 5 mM EDTA, boiled in an equal volume of sample buffer containing 10% 2-mercaptoethanol, and identified by SDS–PAGE on a 10% acrylamide gel using the silver stain technique. (a) Intact TSP; (b) unbound intact TSP; (c) eluted intact TSP; (d) thermolysin-digested TSP; (e) unbound digested TSP; (f) eluted digested TSP.

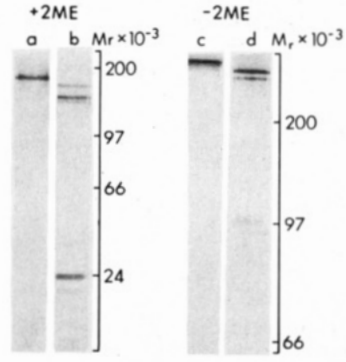

FIGURE 5: SDS–PAGE of intact TSP and thermolytic digest of TSP under reducing and nonreducing conditions. Electrophoresis under reducing conditions was performed on 10% polyacrylamide gels: (a) intact TSP; (b) thermolytic digest of TSP. Electrophoresis under nonreducing conditions was performed on 5% polyacrylamide gels: (c) intact TSP; (d) thermolytic digest of TSP.

from the extreme amino-terminal region of TSP and contains a high-affinity heparin binding site (Dixit et al., 1984b; Coligan & Slayter, 1984).

Intact TSP and a thermolytic digest of TSP were each incubated with fixed, thrombin-activated platelets in TBS containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$. After being washed by centrifugation, the platelet-bound TSP or TSP fragments were eluted by the addition of EDTA-containing buffer, the platelets were removed by centrifugation, and the eluted material was identified by SDS–PAGE. The results are shown in Figure 4. As expected, intact TSP was bound and subsequently eluted from the fixed, activated platelets. When the thermolytic fragments were examined, the M_r 140 000 fragment was the major species bound and subsequently eluted. Relative to the M_r 140 000 fragment, only trace quantities of the M_r 120 000 fragment bound. Little or no binding of the M_r 25 000 fragment was detected.

Origin of the M_r 140 000 Homotrimer. We have recently described a fibrinogen binding region of TSP derived from thermolytic digests of TSP (Dixit et al., 1984). This domain was composed of both 120 000- and 140 000-dalton fragments and was derived from the disulfide-bonded core of the molecule. As shown above, only the M_r 140 000 thermolytic fragment was bound to and subsequently eluted from the fixed, activated platelets. At least two possible explanations exist. The trimeric fragments may exist as heterogeneous forms in which the number of M_r 140 000 chains per trimer varies from zero to three. In this case, only the species containing three M_r 140 000 chains binds to the platelet, presumably because all three binding sites must be employed to generate high-affinity binding. Alternatively, the thermolytic digest might contain predominantly two homotrimeric species composed of either three M_r 140 000 polypeptide chains or three M_r 120 000 polypeptide chains. In this case, only the trimer composed of M_r 140 000 chains would contain the required binding sites.

To clarify the issue, a themolytic digest of TSP was examined by SDS–PAGE under both reducing and nonreducing conditions. If only two discrete homotrimers are present in the digest, only two high molecular weight bands should be observed. If, on the other hand, an array of fragments containing zero to three M_r 140 000 chains is present, four discrete bands or a very broad band due to incomplete resolution of the different species should be observed. In fact, when examined on a 5% polyacrylamide gel under nonreducing conditions, only two discrete high molecular weight bands were present in the digest (Figure 5). Therefore, it appears that the disulfide-bonded, trimeric, high molecular weight fragments produced by thermolysin are composed primarily of either three M_r 120 000 polypeptide chains or three M_r 140 000 polypeptide chains. Apparently, the initial cleavage to produce an M_r 120 000 fragment from an M_r 140 000 homotrimer is rate limiting. Following this initial proteolytic event, cleavage of the second and third chains occurs rapidly to produce the M_r 120 000 homotrimer. The minor species present in Figure 5 under nonreducing conditions apparently represents a trimeric form of the minor species of M_r 35 000 under reducing conditions. This entity has not yet been characterized.

Discussion

When platelets are activated, TSP is secreted from the α-granules of platelets and in the presence of divalent cations becomes associated with the platelet surface (Baenziger et al., 1971). TSP has been identified as mediating the endogenous lectin-like activity expressed by activated platelets (Gartner et al., 1977; Jaffe et al., 1982). Inhibitors of the lectin-like activity of TSP have been shown to inhibit thrombin-induced platelet aggregation (Gartner et al., 1978; Agam et al., 1984), thus implicating TSP as a mediator of platelet aggregation.

The studies described in this report characterize the interaction of TSP with the platelet surface. Several points are established. (1) Human platelets fixed with formaldehyde following thrombin activation are agglutinated by exogenously added TSP. (2) Optimal expression of the platelet agglutinating activity is observed in the simultaneous presence of 2 mM Mg^{2+} and 2 mM Ca^{2+}. (3) The platelet agglutinating activity is inhibited by reduction of the TSP trimer into its M_r 180 000 subunits. (4) Both fibrinogen and heparin are capable of partially inhibiting the platelet agglutinating activity of TSP. Other proteoglycans tested were ineffective or only weakly inhibitory. (5) A thermolytic fragment with M_r 140 000 retains platelet binding activity. An M_r 120 000 fragment previously shown to be derived from the M_r 140 000 fragment (Dixit et al., 1984b) binds only weakly to platelets.

It is also apparent that the endogenous platelet TSP which is secreted upon thrombin activation is insufficient to support platelet agglutination in the absence of exogenously added TSP under the conditions of the experiments described herein. At least two possible explanations exist. First, since the thrombin activation step is conducted in the absence of any added Ca^{2+}, the surface density of endogenous platelet-bound TSP may be below that required for agglutination. Second, the agglu-

tinating activity of endogenous platelet-bound TSP may be reduced by the formaldehyde fixation step.

For reasons which are not apparent, the present result is different from that obtained by Jaffe et al. (1982), who noted that their preparations of fixed, activated platelets aggregated in the absence of added TSP. This finding, however, is difficult to rationalize since their platelet preparations were washed, activated, and washed again in EDTA-containing buffers prior to fixation. Since the binding of fibrinogen (Bennett & Vilaire, 1979; Marguerie et al., 1979), fibronectin (Plow & Ginsberg, 1981), von Willebrand factor (Fujimoto et al., 1982), and TSP (Phillips et al., 1980) to the surface of activated platelets is divalent cation dependent, it is not clear why platelets whose surfaces are devoid of platelet-adhesive proteins should aggregate.

The platelet agglutinating activity of TSP is greatly enhanced when assayed in the presence of both Ca^{2+} and Mg^{2+}. A similar observation has recently been made in our laboratories regarding the hemagglutinating activity of purified TSP (Haverstick et al., 1984). The presence of Ca^{2+}-dependent structural elements within TSP has been well documented (Lawler et al., 1982; Lawler & Simons, 1983). No data have appeared yet demonstrating Mg^{2+}-dependent structures within TSP. We, therefore, cannot yet establish whether Mg^{2+} is required for the structural integrity of TSP or plays a more direct role in the binding of TSP to the platelet surface. The findings described in this report do not preclude the possibility that the self-association of TSP may be altered by the presence of different divalent cations and that such self-association may augment the hemagglutinating and platelet agglutinating activity of TSP.

The TSP molecule is composed of three apparently identical polypeptide chains linked by disulfide bonds (Baenziger et al., 1972; Lawler et al., 1978; Margossian et al., 1981; Dixit et al., 1984b). It seemed likely that this multivalent structure would be essential for the expression of platelet agglutinating activity. This hypothesis is supported by the observation that reduction of TSP disulfide bonds abolishes the platelet agglutinating activity of TSP. It is not possible to rule out that the observed inhibition is due, at least in part, to reduction of intrachain disulfide bonds within structural elements of the TSP molecule required for interaction with the platelet surface. Consistent with this postulate is the observation that even after the interchain disulfide bonds have been completely reduced by SDS–PAGE criteria, some platelet agglutinating activity remains. This activity is only abolished at higher concentrations of the reducing agent. These observations suggest that after reduction just sufficient to reduce the interchain disulfide bonds, the individual polypeptide chains of TSP may still be able to associate under nondenaturing conditions. In our earlier study of the hemagglutinating activity of TSP we observed a closer correlation between the extent of reduction of the interpeptide chain disulfide bonds and the loss of hemagglutinating activity.

Fibrinogen clearly plays a major role in bridging adjacent platelets during platelet aggregation. Our current level of understanding is that upon platelet activation the membrane glycoprotein IIb/III complex becomes able to bind the bivalent fibrinogen molecule, thus forming links between adjacent platelets (Berndt & Phillips, 1981). Platelet-bound fibrinogen has been proposed as the receptor for TSP (Gartner et al., 1981b). As discussed below, two observations in the present study are germane to this proposal.

The presence of 0.25 mg/mL fibrinogen increased the amount of TSP required to produce detectable platelet agglutination from 1.6 to 13 μg/mL, an 8-fold increase in the amount of TSP required. However, further increases in the fibrinogen concentration did not produce any further increase in the TSP requirement. Thus, fibrinogen is only capable of partially inhibiting the platelet agglutinating activity of TSP. In our earlier studies of the hemagglutinating activity of TSP, fibrinogen was observed to have no inhibitory activity whereas heparin was found to be a potent inhibitor (Haverstick et al., 1984). In the present study, heparin was only partially inhibitory.

The divalent cation requirement of the TSP–platelet interaction was exploited to identify the region of the TSP molecule which binds to the platelet. TSP which bound to the surface of fixed, activated platelets could be subsequently eluted by resuspending the platelets in EDTA-containing buffer. TSP could only be bound and subsequently eluted from platelets which had been activated with thrombin prior to fixation; fixed, nonactivated platelets did not bind TSP in a divalent cation dependent manner.

When TSP is subjected to digestion with thermolysin, three major fragments are produced. A polypeptide of M_r 25 000 is derived from the amino terminus of TSP and contains a high-affinity heparin binding site (Dixit et al., 1984b). Two higher molecular weight fragments of M_r 140 000 and 120 000 are also produced. Both of these fragments contain a binding site for fibrinogen (Dixit et al., 1984a). The M_r 140 000 fragment was observed to bind to the surface of activated platelets. Relative to the M_r 140 000 fragment, only trace quantities of the M_r 120 000 fragment were bound even though both fragments were initially present in approximately equimolar concentrations.

These observations suggest that if as proposed by Gartner et al. (1981b) platelet-bound fibrinogen is necessary for the binding of TSP, an additional platelet surface component is also required since the presence of a fibrinogen binding site alone in proteolytic fragments of TSP does not result in a significant degree of binding to the surface of activated platelets. The nature of the additional platelet surface component to which TSP binds remains to be established. The approximately 20 000-dalton fragment of TSP present in the M_r 140 000 polypeptide but not present in the M_r 120 000 fragment derived from the M_r 140 000 polypeptide is presumably required for interaction with this component. The 20 000-dalton fragment is apparently quite unstable and has never been detected in more than trace quantities. Interestingly, this same region of the TSP molecule is required for binding to trypsinized red cells (Haverstick et al., 1984). Since red blood cells have not been shown to have surface-bound fibrinogen, this provides further support for the suggestion that TSP–platelet interactions are not mediated solely by fibrinogen.

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