Localization of the Hemagglutinating Activity of Platelet Thrombospondin to a 140 000-Dalton Thermolytic Fragment†

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ABSTRACT: The platelet protein thrombospondin (TSP) which is secreted from α-granules upon platelet activation agglutinates trypsinized, glutaraldehyde-fixed human erythrocytes. Optimal conditions for the hemagglutinating activity require that both Ca^{2+} and Mg^{2+} be present in final concentrations of 2 mM. In the presence of dithiothreitol (i.e., reduction of disulfide bonds), the lectin-like activity decreases in a manner proportional to the extent of reduction of the molecule from its native trimeric configuration into its M_r 180 000 subunits. Proteolysis of purified TSP with thermolysin, which produces discrete domains with the capacity to bind fibrinogen and heparin, also diminishes, but does not abolish, the hemagglutinating activity. Fibrinogen was without effect on hemagglutinating activity while heparin was found to be a potent inhibitor. Other proteoglycans such as hyaluronic acid, chondroitin sulfate, keratan sulfate, dermatan sulfate, and heparan sulfate had no effect. That portion of the TSP molecule apparently responsible for the hemagglutinating activity was identified by incubating a thermolytic digest of TSP with red blood cells and then determining which fragment was bound to the cell surface. The binding site resides within a peptide fragment of 140 000 daltons but is absent from an M_r 120 000 fragment derived from the M_r 140 000 fragment. Under the conditions for optimal expression of hemagglutinating activity (i.e., 2 mM $MgCl_2$ and 2 mM $CaCl_2$), this M_r 140 000 fragment was also shown to have heparin binding activity.

Following activation by thrombin, platelets agglutinate glutaraldehyde-fixed, trypsinized erythrocytes via the expression of an endogenous lectin-like activity on their surface (Gartner et al., 1977). Jaffe et al. (1982) subsequently demonstrated that the protein secreted from platelet α-granules, thrombospondin, which has also been referred to as thrombin-sensitive protein (TSP)[1] (Baenziger et al., 1971, 1972) or glycoprotein G (Phillips et al., 1980), is the endogenous lectin which mediates the hemagglutinating activity. TSP was originally described by Baenziger et al. (1971, 1972), who noted the absence of the high molecular weight glycoprotein from platelets after activation with thrombin and its association with platelet membrane fractions. Subsequent investigations revealed that TSP is located within the α-granules and is secreted upon platelet activation (Gartner et al., 1981a). It has since been shown that the protein is also synthesized and secreted from 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). The synthesis and incorporation of TSP into extracellular matrices of these diverse cell types suggest an important role for the protein in cell–cell and cell–substratum interactions and have stimulated new interest in the molecule.

The protein is a trimer composed of subunits of M_r 180 000 that are disulfide bonded (Baenziger et al., 1972; Lawler et al., 1978; Margossian et al., 1981). TSP has recently been shown to have Ca^{2+}-sensitive structures (Lawler & Simons, 1983; Lawler et al., 1982) and to display the capacity to bind to fibrinogen, fibronectin, collagen, and heparin (Lawler et al., 1978; Leung & Nachman, 1982; Lahav et al., 1982). Recent work (Lawler & Slayter, 1981; Mumby et al., 1984; Dixit et al., 1984a) has shown that these binding functions are located within discrete regions of the molecule, which can be isolated from limited proteolytic digests of the intact molecule by affinity chromatography. In this respect, the molecule may be similar to the extracellular matrix protein fibronectin (Hynes & Yamada, 1982) which is also composed of discrete domains with specific binding activities.

On the basis of this identification of discrete domains within the TSP molecule, we have further extended our investigations to charcterize the hemagglutinating activity of the protein. It is possible to correlate the known binding specificities of TSP with the lectin-like activity expressed by the protein and to identify the region of the molecule responsible for mediating red cell agglutination. Since inhibitors of the hemagglutining activity have been reported to inhibit thrombin-induced platelet aggregation (Gartner et al., 1978), such studies may be relevant to the mechanism of platelet–platelet interactions.

Materials and Methods

Materials. Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Beef lung heparin was obtained from Upjohn (Kalamazoo, MI), and porcine mucosal heparin was obtained from Sigma. Both preparations behaved identically in the hemagglutination assay. Human thrombin was a generous gift from Dr. Joseph P. Miletich, Washington University School of Medicine, and heparan sulfate was provided by Dr. Alfred Linker, University of Utah. Fibrinogen (grade L) was from Kabi (Sweden).

Purification of TSP. The purification of TSP from platelet concentrates in Ca^{2+}-replete form was based on a modification

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[1] Abbreviations: TSP, thrombospondin; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; M_r, apparent molecular weight; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; TBS, Tris-buffered saline.

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of the method of Margossian et al. (1981). Briefly, washed platelets were activated by the addition of human thrombin (2 units/mL), the reaction was stopped by the addition of hirudin (4 units/mL) and PMSF (2 mM), and platelet aggregates and fibrillar material were removed by centrifugation. The resulting supernatant was subjected to chromatography on gelatin–Sepharose (to remove contaminating fibronectin) followed by chromatography on heparin–Sepharose. Both columns were equilibrated with 0.02 M Tris (pH 7.6), 0.15 M NaCl, and 1 mM $CaCl_2$ at 4 °C. TSP was eluted from the latter column by increasing the NaCl concentration to 0.6 M and then separated from lower molecular weight contaminants by gel filtration over a Bio-Gel A 0.5M column (2 × 100 cm) equilibrated with 0.02 M Tris (pH 7.6), 0.15 M NaCl, and 1 mM $CaCl_2$ at 4 °C. The isolated TSP, free of any contaminating material by SDS–PAGE criteria, was stored at −70 °C in 0.02 M Tris, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, and 20% (w/v) sucrose prior to use.

Assay for Hemagglutinating Activity. The procedure is based on the method of Gartner et al. (1977). Assays were carried out in microtiter "V" plates (Dynatech Laboratories, Inc., Alexandria, VA) in a final volume of 100 μL. Serial dilutions of TSP in 0.05 M Tris (pH 7.4) and 0.15 M NaCl were made in 25 μL directly in the microtiter plates. Since preliminary experiments revealed that the hemagglutination assay was unaffected by diluted sucrose solutions, sucrose was not routinely removed from the TSP solutions. Fifty microliters of the final volume was made up of buffer containing any salts under examination, the domains of the TSP digest, possible inhibitors, etc., as conditions for the individual experiment dictated. For the data presented in Tables III and V, the TSP concentration was kept constant, and the proteoglycans were serially diluted. Unless otherwise indicated, all assays were conducted in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$ (see Results). The assay was initiated by the addition of 25 μL of a 2.5% suspension of trypsinized, glutaraldehyde-fixed human erythrocytes purchased from Calbiochem-Behring (La Jolla, CA). The microtiter plate was agitated for 1 h at room temperature on a Hyperion (Miama, FL) micromix shaker and then examined for agglutination of the red blood cells. The results are presented as the minimum concentrations of TSP capable of causing detectable agglutination of the cells, except in the cases of Tables III and V, where the minimum concentration of the proteoglycan causing inhibition of the TSP-induced agglutination is reported. To ascertain that the hemagglutinating activity that we observed was the same as that reported by Gartner et al. (1977), we tested for inhibition of activity by the simple sugars employed in that study and found an identical pattern of inhibition.

SDS–PAGE. Polyacrylamide gel electrophoresis was carried out in a slab gel apparatus using the discontinuous gel system described by Laemmli (1970). Protein was identified on the gels by the silver stain method using either the kit obtained from Bio-Rad, Inc. (Richmond, CA), or the system described by Merrill et al. (1981).

Proteolysis 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/μg of thermolysin. The digest was used without further manipulation or for some experiments fractionated by chromatography on heparin–agarose. The digest was applied to a 2-mL column of heparin–agarose equilibrated with 0.02 M Tris, pH 7.4, 0.15 M NaCl, and 1 mM $CaCl_2$ at 4 °C.

Table I: Divalent Cation Requirements for Expression of TSP-Induced Hemagglutinating Activity

conditions	min eff TSP concn[a] (μg/mL)	conditions	min eff TSP concn[a] (μg/mL)
TBS[b]	>125	TBS	
+5 mM EDTA	>125	+2 mM $CaCl_2$ + 2 mM $MgCl_2$	4
+1 mM $CaCl_2$	>125		
+1 mM $MgCl_2$	63	+4 mM $CaCl_2$	125
+1 mM $CaCl_2$ + 1 mM $MgCl_2$	31	+4 mM $MgCl_2$	31
		+5 mM $CaCl_2$ + 5 mM $MgCl_2$	4

[a] Minimum effective concentration of TSP causing hemagglutination. [b] 0.05 M Tris (pH 7.4) and 0.15 M NaCl.

After the column was extensively washed, bound fragments were eluted by increasing the NaCl concentration to 0.75 M.

Protein Determinations. Protein was quantitated by the method by Lowry et al. (1951). Bovine serum albumin was employed as a standard.

Results

Divalent Cation Dependence of Hemagglutination. On the basis of the recent demonstration of Ca^{2+}-sensitive structures within the TSP molecule (Lawler et al., 1982; Lawler & Simons, 1983) and our recent demonstration that both Ca^{2+} and Mg^{2+} are required for optimal binding of TSP to fibrinogen (Dixit et al., 1984a), we first sought to establish the divalent cation requirements for optimal expression of the hemagglutinating activity of highly purified Ca^{2+}-replete TSP. The results (Table I) show that the hemagglutinating activity is exquisitely sensitive to divalent cations. The presence of both Mg^{2+} and Ca^{2+}, each in a final concentration of 2 mM, provided optimal activity. Table I also shows that neither cation alone at 4 mM could effectively substitute for the combination nor could the hemagglutinating activity of TSP be enhanced by further increases in the Mg^{2+} or Ca^{2+} concentrations above 2 mM. On the basis of these results, all further assays were carried out in a system containing (final concentration) 2 mM $MgCl_2$ and 2 mM $CaCl_2$, unless otherwise specified. More detailed analyses to further refine the optimal divalent cation requirements have not been performed.

Reduction of Disulfide Bonds Inhibits Hemagglutinin Activity. Since TSP is known to be a disulfide-bonded trimer of subunit M_r 180 000 (Baenziger et al., 1972; Lawler et al., 1978; Margossian et al., 1981), the effect of disulfide bond reduction on the hemagglutinating activity was investigated by first incubating the intact molecule with dithiothreitol (DTT) and then examining the hemagglutinating activity of the partially and fully reduced preparations. As shown in Figure 1, increasing the concentration of the reducing agent caused the appearance of the M_r 180000 band on SDS–PAGE with a corresponding decrease in the band of high molecular weight at the top of the gel representing the intact trimeric molecule. At 5 mM DTT, the molecule is partially reduced, and by 25 mM DTT, the band corresponding to the intact trimeric molecule is undetectable. This reduction in molecular weight is paralleled by a reduction in the hemagglutinating activity of TSP. At 10 mM DTT, there is an 8-fold increase in the minimum amount of TSP needed to cause detectable agglutination, and after reduction with 25 mM DTT, 96 μg/mL TSP is not sufficient to cause hemagglutination.

Effect of Thermolytic Digestion on TSP-Induced Hemagglutination. TSP appears to be composed of discrete, relatively protease-resistant domains linked by regions which are more susceptible to proteolytic cleavage. In this regard, TSP may be similar to the well-characterized plasma, cell-surface,

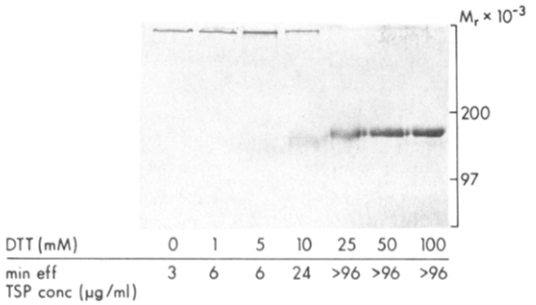

FIGURE 1: Effect of disulfide bond reduction on the hemagglutinating activity of TSP. TSP (45 μg) was incubated with the indicated dithiothreitol (DTT) concentrations at 37 °C for 1 h. One aliquot of each sample was then diluted with SDS sample buffer in the absence of any further reducing agent and boiled for 5 min. 15 μg of protein in 100 μL was loaded onto a 6% polyacrylamide gel. Bands were identified by the silver stain method. An additional aliquot of each sample was serially diluted and tested for hemagglutinating activity. The minimum concentration of each sample capable of producing hemagglutination is indicated below the corresponding lane of the gel.

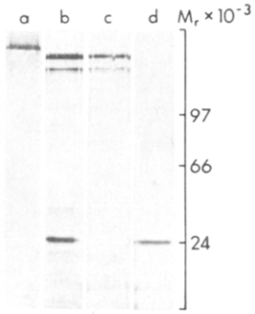

FIGURE 2: SDS–PAGE of isolated thermolytic fragments of TSP. The thermolysin digest of TSP (1:100 w/w for 1 h) was subjected to affinity chromatography on heparin–agarose. Representative samples were boiled in SDS sample buffer in the presence of 2-mercaptoethanol and applied to a 10% polyacrylamide gel. Protein bands were identified by silver staining. (Lane a) Intact TSP; (lane b) thermolysin digest of TSP; (lane c) unbound fragments; (lane d) fragment eluted with 0.75 M NaCl.

and extracellular matrix protein fibronectin (Hynes & Yamada, 1982). When Ca^{2+}-replete TSP is subjected to digestion with thermolysin at an enzyme:substrate ratio of 1:100, three major fragments with molecular weights of 140 000, 120 000, and 25 000 are produced (Figure 2, lane b). The larger fragments which are derived from the disulfide-bonded core region of the molecule constitute the fibrinogen binding domain (Dixit et al., 1984a). The smaller 25 000-dalton fragment is not linked by interchain disulfide bonds and represents a high-affinity heparin binding domain (Lawler & Slayter, 1981; Dixit et al., 1984b).

Purified TSP was subjected to digestion with thermolysin under the above conditions and the digest examined for hemagglutinating activity. Whereas the intact TSP molecule was capable of producing hemagglutination at concentrations as low as 1.6 μg/mL, following digestion with thermolysin an almost 40-fold increase in the TSP concentration (50 μg/mL) was required to produce hemagglutination. No intact TSP was demonstrable by SDS–PAGE following digestion with thermolysin (Figure 2, lane b). These results indicate that the proteolytic digestion disrupted a region of TSP structure necessary for the optimal expression of the lectin-like activity. However, while proteolytic digestion greatly diminishes the hemagglutinating activity of TSP, it does not completely abolish all detectable activity as did disulfide bond reduction.

Heparin Inhibits Hemagglutinin Activity. As described above, distinct heparin binding and fibrinogen binding polypeptides have been isolated from thermolytic digests of TSP (Dixit et al., 1984a,b). Fibrinogen and heparin were therefore examined for effects on the hemagglutinating activity of TSP. At concentrations up to 50 μg/mL, fibrinogen was found to have no effect on the hemagglutination induced by 0.8 μg/mL TSP (data not shown). Heparin, on the other hand, had a very marked inhibitory effect (Table II). In the absence of heparin, TSP caused agglutination at a concentration of 1.4 μg/mL. The addition of as little as 0.08 unit of heparin/mL (0.5 μg/mL), however, necessitated an 8-fold increase in the amount of TSP necessary to cause agglutination. Higher concentrations of heparin resulted in further increases in the minimum concentration of TSP which would produce hemagglutination (Table II). Since heparin is a large, highly charged proteoglycan, the possibility that the observed inhibition of hemagglutination resulted simply from the presence of such a macromolecule in the assay had to be considered. This possibility seems unlikely, however, since the hemagglutinating activity of wheat germ agglutinin was unaffected by the presence of 1 unit/mL heparin (data not shown). Furthermore, as shown in Table III, when other proteoglycans such as chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and hyaluronic acid were examined, no significant inhibition of TSP-induced hemagglutination was observed. The striking inhibitory activity of heparin is demonstrated by the ability of 4.5 μg/mL heparin to inhibit the hemagglutinating activity of 38 μg/mL TSP. This inhibition is several orders of magnitude greater than that previously reported for monosaccharides and amino acids (Gartner et al., 1977). Other proteoglycans when present at concentrations exceeding 1–2 mg/mL were unable to inhibit the lectin activity of only 7 μg/mL TSP.

Effect of TSP Fragments on Hemagglutinating Activity. In an initial attempt to localize the hemagglutinating activity of TSP, the two major domains of the TSP molecule, the 25 000-dalton heparin binding fragment and the 120 000–140 000-dalton fibrinogen binding fragment, were tested for their ability to compete with TSP in the agglutination reaction. The fragments were isolated from thermolytic digests of TSP

Table II: Heparin Inhibition of TSP-Induced Hemagglutinating Activity

[heparin] (units/mL)[a]	min eff TSP concn[b] (μg/mL)	[heparin] (units/mL)[a]	min eff TSP concn[b] (μg/mL)
0	1.4	0.63	23
0.08	12	1.25	46
0.31	23	2.50	46

[a] Specific activity of heparin was 170 units/mg. [b] Minimum effective concentration of TSP causing hemagglutination.

Table III: Effect of Proteoglycans on TSP-Induced Hemagglutinating Activity[a]

proteoglycan	min inhibitory concn (μg/mL) TSP (38 μg/mL)	TSP (7 μg/mL)
heparin	5	3
hyaluronic acid	1250	>1250
chondroitin sulfate	>2500	>2500
dermatan sulfate	>2500	1250
keratan sulfate	2500	>1250
heparan sulfate	>2500	>2500

[a] TSP was tested for hemagglutinating activity in the presence of the serially diluted proteoglycans listed. The results are reported as the minimum concentration of the proteoglycan that prevented detectable hemagglutination.

Table IV: Effect of Isolated TSP Fragments on TSP-Induced Hemagglutination

conditions	min eff TSP concn[a] (μg/mL)
TSP alone	1.25
TSP plus M_r 120000–140000 fragments (30 μg/mL)	0.30
TSP plus M_r 25000 fragment (30 μg/mL)	2.50

[a] Minimum effective concentration of TSP causing hemagglutination

Table V: Ability of Isolated Fragments of TSP To Overcome Heparin Inhibition of TSP-Induced Hemagglutination[a]

conditions	min inhibitory concn (units/mL)
TSP	0.08
TSP plus M_r 120000–140000 fragment (20 μg/mL)	>5
TSP plus M_r 25000 fragment (22 μg/mL)	>5

[a] Heparin was serially diluted and tested for the ability to inhibit the hemagglutination produced by 2 μg/mL TSP under the conditions indicated.

by chromatography of the digest on a heparin–agarose affinity column equilibrated in 0.02 M Tris, pH 7.4, 0.15 M NaCl, and 1 mM $CaCl_2$. As shown in Figure 2, the larger molecular weight fibrinogen binding region of TSP passes through the column without binding (lane c) whereas the smaller M_r 25000 high-affinity heparin binding domain binds to the column (band absent in lane c) and is eluted from the column by the addition of 0.6 M NaCl to the column buffer (lane d). After dialysis against the column buffer, these two domains were then added to the hemagglutination assay system at a concentration of 30 μg/mL to determine if they had an effect on TSP-mediated hemagglutination. As seen in Table IV, the 120000–140000-dalton fibrinogen binding fragment appeared to potentiate the ability of 2 μg/mL intact TSP to agglutinate the fixed erythrocytes. In the presence of these high molecular weight fragments, there was a 75% reduction in the amount of intact TSP required to produce agglutination. At the concentration employed (30 μg/mL), the purified M_r 120000–140000 fragment did not produce agglutination in the absence of intact TSP (data not shown). The M_r 25000 heparin binding domain caused at most a very slight inhibition of the agglutination reaction (at no time was a shift of more than one well in the microtiter plate observed) and was considered to be without an effect.

Thermolytic Fragments of TSP Neutralize Inhibitory Activity of Heparin. The capacity for one or both of the thermolytic fragments to compete with TSP for heparin and hence overcome the inhibition of the hemagglutinating activity by heparin was investigated. The effectiveness of heparin as an inhibitor of TSP-induced hemagglutination was examined in the presence of TSP alone or in the presence of TSP and either of the two major thermolytic domains. As shown in Table V, 0.08 unit/mL heparin effectively inhibited the hemagglutination induced by 2 μg/mL TSP. In the presence of either the M_r 120000–140000 fibrinogen binding domain (20 μg/mL), the hemagglutinating activity of 2 μg/mL TSP was not inhibited by concentrations of heparin up to 5 units/mL. These results suggest that in addition to the high-affinity heparin binding site previously characterized within the 25000-dalton fragment an additional heparin binding site might also be present in the M_r 120000–140000 fragment(s). Intact TSP, but not other irrelevant proteins such as bovine serum albumin, ovalbumin or gelatin, could overcome the heparin inhibition when present at 20 μg/mL.

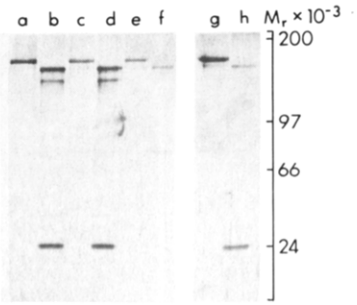

FIGURE 3: SDS–PAGE identification of the red cell binding fragment of TSP. (Lanes a–f) Intact TSP (lane a) and the thermolysin digest of TSP (lane b) were incubated with glutaraldehyde-fixed, trypsinized red blood cells in 0.05 M Tris (pH 7.4), 0.15 M NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. The cells were centrifuged and washed in the above buffer. Bound fragments were eluted with 0.05 M Tris (pH 7.4), 0.15 M NaCl, and 5 mM EDTA. The supernatants were boiled in SDS sample buffer containing 2-mercaptoethanol and applied to a 10% polyacrylamide gel. Proteins were detected by staining with silver nitrate. (Lane c) Unbound intact TSP; (lane d) unbound TSP digest; (lane e) EDTA-eluted intact TSP; (lane f) EDTA-eluted TSP digest. (Lanes g and h) Intact TSP and digested TSP were incubated with heparin–agarose under the same conditions as in the experiment described for lanes a–f. The heparin–agarose was centrifuged and washed in the binding buffer and then eluted with 0.05 M Tris (pH 7.4) plus 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.75 M NaCl. (Lane g) Material eluted from heparin–agarose incubated with intact TSP; (lane h) material eluted from heparin–agarose incubated with the TSP digest.

Localization of the Red Cell Binding Site of TSP. In an effort to determine directly which of the major thermolytic fragments of TSP mediates the red cell agglutination, glutaraldehyde-fixed, trypsinized human erythrocytes were washed and resuspended in 0.05 M Tris (pH 7.4), 0.15 M NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. The cells were then incubated for 30 min at room temperature with intact TSP or a thermolysin digest of TSP. The cells were centrifuged and washed in the above incubation buffer. The requirement for Ca^{2+} and Mg^{2+} was then exploited to elute bound material from the red cells by resuspending them in 0.05 M Tris (pH 7.4) and 0.15 M NaCl containing 5 mM EDTA to elute any material bound to the cell surface in the presence of Mg^{2+} and Ca^{2+}. The cells were then removed by centrifugation, and any EDTA-eluted material in the supernatant was identified by silver staining following separation by SDS–PAGE. The results are shown in Figure 3. There is clearly an excess of TSP or TSP fragments in the assay system, on the basis of the continued presence of the intact molecule (lane c) and the appearance of all bands of the digest in lane d (to be compared to lane b) in the unbound fractions. The addition of 5 mM EDTA cleanly elutes the bound intact TSP from the washed erythrocytes (lane e) and elutes a band of M_r 140000 from those cells incubated with the TSP digest (lane f). Thus, the erythrocyte binding site is apparently located within the M_r 140000 thermolytic fragment of TSP. Since the M_r 140000 and 120000 fragments were present in nearly equal amounts in the original digest, it is clear that the M_r 140000 fragment has a significantly greater affinity for the red cell than does the M_r 120000 fragment.

Heparin Binding Activity of the M_r 140000 Fragment. To ascertain whether or not the M_r 140000 thermolytic fragment of TSP also possessed heparin binding activity which would account for the ability of the 120000–140000-dalton fragment to overcome the inhibition of TSP-induced hemagglutination by heparin, intact TSP or thermolytic digests of TSP were incubated with heparin–agarose at room temperature in 0.05

M Tris (pH 7.4), 0.15 M NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. The resin was washed by centrifugation and any bound material subsequently eluted by increasing the NaCl concentration to 0.75 M. As shown in Figure 3 (lanes g and h), intact TSP, the previously described M_r 25 000 high-affinity heparin binding domain, and the M_r 140 000 fragment were all bound to and eluted from heparin–agarose. The M_r 120 000 fragment was not bound to any significant extent.

Discussion

Thrombospondin was originally described by Baenziger et al. (1971) as a thrombin-sensitive glycoprotein associated with human platelet membranes. Subsequent workers (Gartner et al., 1981a; Phillips et al., 1980; Lawler et al., 1978) showed that TSP is secreted from α-granules upon activation of platelets by thrombin or the calcium ionophore A23187. In the presence of divalent cations, TSP becomes associated with the platelet membrane (Phillips et al., 1980). Recent evidence suggests that fibrinogen bound to the platelet membrane glycoprotein IIb–glycoprotein III complex is the TSP receptor (Leung & Nachman, 1982; Gartner et al., 1981b).

TSP has been shown to be the protein responsible for the lectin-like activity of platelets which is thought to mediate platelet aggregation and erythrocyte agglutination (Gartner et al., 1978; Jaffe et al., 1982). However, the discovery that it is secreted by and incorporated into the extracellular matrix of several cell lines in culture (McPherson et al., 1981; Mosher et al., 1982; Raugi et al., 1982; Jaffe et al., 1983; Sage et al., 1983) suggests that the protein may have a broader role in cell–cell and cell–substrate interactions. As yet, no clear-cut physiological role for the protein has been described, although the molecule has been shown to be capable of binding to heparin, fibrinogen, fibronectin, and collagen (Lawler et al., 1978; Lawler & Slayter, 1981; Leung & Nachman, 1982; Lahav et al., 1982; Mumby et al., 1984; Dixit et al., 1984a). These activities and other properties of TSP suggest a similarity to fibronectin, further supporting an analogous functional role for TSP.

The studies described in this report establish several points regarding the hemagglutinating activity of highly purified platelet thrombospondin: (a) Optimal expression of the lectin-like activity requires the presence of both Ca^{2+} and Mg^{2+}. (b) The disulfide-bonded trimeric structure of TSP is necessary for hemagglutinating activity; reduction of interchain disulfide bonds is accompanied by a parallel loss of lectin-like activity. (c) Limited proteolysis results in the diminution but not elimination of the hemagglutinating activity of TSP. (d) The high molecular weight fragments are capable of potentiating the lectin-like activity of intact TSP. (e) Heparin is a potent inhibitor of TSP-induced hemagglutination while other proteoglycans are not effective inhibitors. (f) Both high and low molecular weight proteolytic fragments of TSP are capable of neutralizing the inhibitory activity of heparin. (g) By exploiting the divalent cation requirement for TSP-induced hemagglutination, it has been possible to identify proteolytic fragments of M_r 140 000 which are capable of binding to the red cell surface. Other fragments of only slightly lower molecular weight, M_r 120 000, do not retain this activity.

Evidence supporting the presence of Ca^{2+}-sensitive structures within the TSP molecule has recently appeared (Lawler et al., 1982; Lawler & Simons, 1983). It is important to note, therefore, that the TSP used in the present investigation has been purified in a Ca^{2+}-replete form. The data in Table I clearly indicate that both Ca^{2+} and Mg^{2+}, each at 2 mM, are required for optimal hemagglutinating activity. Higher concentrations of either divalent cation alone will not substitute for the combination nor will further increases in the concentration of Ca^{2+} and Mg^{2+} together above 2 mM further enhance the hemagglutinating activity. These results suggest that in addition to the Ca^{2+}-sensitive structures defined earlier, there may exist additional structural elements which depend upon Mg^{2+} for maintenance. Alternatively, either Ca^{2+} or Mg^{2+} could be directly involved in the interactions of TSP with the red cell surface. Additional physicochemical and ultrastructural studies are required for resolution of this point.

The TSP molecule is composed of three apparently identical polypeptide chains linked by disulfide bonds. It seemed likely that this multivalent structure would be essential for expression of the lectin-like activity. This hypothesis is supported by the correlation between loss of hemagglutinating activity and reduction of the interpolypeptide chain disulfide bonds. However, we cannot absolutely exlude the possibility that at least some loss of hemagglutinating activity may result from reduction of intrachain disulfide bonds within structural elements of the TSP molecule which interact with the red cell surface. Similarly, we cannot exclude that an effect of divalent cations or sulfhydryl reduction on the possible self-association of TSP may be reflected in an alteration of the hemagglutinating activity of the molecule.

When Ca^{2+}-replete TSP is subjected to limited proteolysis, only a small number of peptides are produced (Lawler & Slayter, 1981; Dixit et al., 1984a; Figure 2). We have extensively characterized the products obtained from limited digestion of TSP with thermolysin. The products include an M_r 25 000 fragment which binds to heparin with high affinity in the absence of divalent cations. This fragment contains no intrachain disulfide bonds and is derived from the amino-terminal region of the TSP polypeptide (Dixit et al., 1984b). Fragments of M_r 120 000 and 140 000 also constitute major products of the thermolytic digest. These fragments emanate from the disulfide-bonded core region of the molecule and bind to fibrinogen when both Ca^{2+} and Mg^{2+} are present (Dixit et al., 1984a). Thus, the regions of divalent cation dependent structure are presumably located within these fragments.

When purified TSP was subjected to limited thermolytic digestion, the hemagglutinating activity was diminished approximately 40-fold, but clearly not abolished. The hemagglutinating activity of intact TSP was not affected by the presence of the purified M_r 25 000 fragment. In contrast, the M_r 120 000–140 000 fragments potentiated the hemagglutinin activity of intact TSP. The concentration of the M_r 120 000–140 000 fragments employed lacked detectable hemagglutinating activity in the absence of intact TSP. These results suggest that the M_r 120 000–140 000 fragments contain the site(s) which interact with the erythrocyte surface.

Since, as noted above, TSP is capable of binding to both fibrinogen and heparin, each of these ligands was examined for any effects on the hemagglutination assay. Fibrinogen had no effect on TSP-induced hemagglutination. In contrast, heparin was found to be a potent inhibitor of TSP-induced hemagglutination. Other proteoglycans were not effective inhibitors of hemagglutination. As noted earlier, the high-affinity heparin binding domain of TSP is located within the M_r 25 000 thermolytic fragment. This fragment, however, had no effect on TSP-induced hemagglutination. The interaction of this domain of TSP with heparin does not require divalent cations (Dixit et al., 1984b). Since the hemagglutinating activity of TSP is dependent upon the presence of both Ca^{2+} and Mg^{2+}, it seems unlikely that the interaction of heparin with the M_r 25 000 domain of TSP accounts for the ability of heparin to inhibit TSP-induced hemagglutination. In

contrast, the loss of hemagglutinating activity upon reduction of interchain disulfide bonds located within the M_r 120000–140000 fragment and the ability of these larger molecular weight fragments to potentiate the hemagglutinating activity of intact TSP suggest that the red cell binding site lies within the M_r 120000–140000 fragments.

Both the M_r 25000 fragment and the M_r 120000–140000 fragments were purified and then tested for ability to overcome the inhibitory activity of heparin in the hemagglutination assay. Surprisingly, both fragments at concentrations of 20–22 μg/mL were effective in neutralizing the inhibitory activity of 5 units/mL heparin. This finding suggests that in addition to the high-affinity heparin binding site previously identified, another heparin binding site may also be present within the M_r 120000–140000 fragment. In fact, during our previous isolation of the 25000-dalton heparin binding domain, extensive washing of the heparin–agarose was required to avoid contamination of the M_r 25000 fragment with the M_r 140000 fragment. It has recently been established that several distinct binding sites with differing affinities for heparin are present within the fibronectin molecule (Hynes & Yamada, 1982) and that the affinities of some of these sites are sensitive to cation modulation (Hayashi & Yamada, 1982).

For direct identification of the location of the red cell binding site within the TSP molecule, a thermolytic digest of TSP was incubated with glutaraldehyde-fixed, trypsinized red cells in the presence of 2 mM concentrations of both Ca^{2+} and Mg^{2+}. After extensive washing, we eluted fragments of TSP which were bound to the red cell in a divalent cation dependent manner by addition of EDTA-containing buffer. Only the M_r 140000 fragment was recovered. Similar binding and elution experiments with TSP fragments and heparin–agarose were conducted except that high concentrations of NaCl rather than EDTA were used to elute bound material. As expected, the M_r 25000 fragment was bound and subsequently eluted. The M_r 140000 fragment was also bound and eluted. The M_r 120000 fragment did not appear to bind. Thus, both a red cell binding site and an additional heparin binding site appear to be located within the 140000-dalton fragment of the thermolytic digest of TSP. It remains to be established whether this second heparin binding site is truly modulated by Mg^{2+} or whether its affinity for heparin is such that the interaction is only marginally stable at physiologic ionic strength and hence it is not recovered with the more extensive washing associated with affinity chromatography. As noted above, precedents for both exist within fibronectin.

Since peptide maps have shown that these two fragments are highly related and the M_r 120000 fragment is derived by proteolysis from the M_r 140000 fragment (Dixit et al., 1984a), it seems likely that structural determinants within either the carboxy- or the amino-terminal 20000 daltons of the M_r 140000 fragment are essential for binding to the erythrocyte surface. Determinants for an additional heparin binding site are also expressed within this region of the molecule. We have previously shown that both the M_r 120000 and M_r 140000 fragments contain the fibrinogen binding site. Presumably, fibrinogen binds at a locus some distance from the red cell binding site since it is not inhibitory in the hemagglutination assay.

The residual hemagglutinating activity of the thermolytic digest and the ability of the purified M_r 120000–140000 fragments (at concentrations below that at which they produce hemagglutination by themselves) to potentiate the hemagglutinating activity of intact TSP presumably arise from species containing at least two M_r 140000 polypeptide chains. Preliminary evidence (D. M. Haverstick and S. A. Santoro, unpublished results) indicates that the digest is composed predominantly of two populations of homotrimers containing three M_r 140000 polypeptides or three M_r 120000 polypeptides.

Attempts to obtain a 20000-dalton fragment cleaved from the M_r 140000 fragment have thus far been unsuccessful. The small fragment may be unstable and subject to additional proteolysis. Additional studies will be required to further localize the red cell binding region of TSP, as well as other physiologically important binding sites on the TSP molecule. It is interesting to note that only recently have small polypeptides of M_r 15000 been prepared from the originally described 140000–160000-dalton cell binding domain of fibronectin (Pierschbacher et al., 1981, 1982). Similar approaches may prove feasible with TSP.

Registry No. Mg, 7439-95-4; Ca, 7440-70-2; heparin, 9005-49-6.

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Purification of Human γ-Interferon to Essential Homogeneity and Its Biochemical Characterization†

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ABSTRACT: A multistep procedure has been developed which enables human γ-interferon (HuIFN-γ) to be purified to essential homogeneity. The procedure takes advantage of a modification of a previously described sequential chromatographic technique [Braude, I. A. (1983) *Prep. Biochem. 13*, 177–190] and the high isoelectric point of HuIFN-γ (pH 9.5–9.8). The steps include Controlled Pore Glass adsorption chromatography, concanavalin A–Sepharose and heparin–Sepharose affinity chromatography, cation-exchange chromatography, and gel filtration chromatography. The purified HuIFN-γ had a specific activity of 5.9×10^7 units/mg. This represents a purification of more than 70 000-fold and a 33% recovery. In addition, one gel filtration fraction had a specific activity of 2.5×10^8 units/mg. This represents a purification of greater than 300 000-fold and a recovery of greater than 17%. This fraction, when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, was shown to be composed of one major 26-kilodalton (kDa) species and four minor species of 74, 67, 56, and 22 kDa. Analysis of this material with anti-HuIFN-γ monoclonal antibody immunoabsorbent columns indicates that both the 26- and the 22-kDa species are HuIFN-γ. Thus, the final product is essentially homogeneous (90–92% HuIFN-γ), and the specific activity of pure HuIFN-γ is approximately $(2.7–2.8) \times 10^8$ units/mg of protein. Finally, the 26- and 22-kDa moieties are shown to be similar, if not identical, proteins as judged by amino acid and sequence analyses.

Interferons (IFN's)[1] are a family of proteins which possess a variety of biological properties. Most notable are its antiviral (Isaacs & Lindenmann, 1957), antiproliferative (Gresser et al., 1970; Strander & Einhorn, 1977), and immunoregulatory (Johnson, 1978; Herberman et al., 1979; Hernandez-Asensio et al., 1979; Minato et al., 1980) characteristics. The most recent interferon class to be discovered is IFN-γ (Wheelock, 1965; Falcoff et al., 1972; Salvin et al., 1973; Epstein, 1976).

Unlike IFN-α and IFN-β, IFN-γ is primarily produced by antigen- or mitogen-stimulated T lymphocytes. IFN-γ has also been shown to be physiochemically (Langford et al., 1979; Yip et al., 1981; Gray et al., 1982) and antigenically (deLey et al., 1980; Wiranowski-Stewart et al., 1980) distinct from IFN-α and IFN-β. However, similar to IFN-α and IFN-β, IFN-γ induces or alters many of the same intracellular metabolic events such as the (2′–5′)-oligoadenylate synthetase (Baglioni & Maroney, 1980) and protein kinase systems (Falcoff et al., 1980), as well as a variety of de novo synthesized proteins (Rubin et al., 1980).

Interest in the IFN-γ system has increased since data describing its potent antiproliferative (deLey et al., 1980; Rubin & Gupta, 1980), immunoregulatory (Basham & Merigan, 1983), and (when used in combination with either IFN-α or IFN-β) potentiating (Fleischmann et al., 1979; De Clercq et al., 1982) properties were reported. In addition, clinical studies to evaluate HuIFN-γ's antitumor properties are being conducted (Gutterman et al., 1983; Oldham et al., 1983).

Further studies regarding the biological and biochemical properties of HuIFN-γ require highly purified preparations. In this report, a procedure is described for the purification of HuIFN-γ to essential homogeneity. Furthermore, employing a variety of immunochemical and biochemical techniques, it is shown that HuIFN-γ is found as at least two molecular weight forms whose polypeptide structures are similar if not identical.

Materials and Methods

Resins. Controlled Pore Glass beads (350-Å pore size, 120–200 mesh) were purchased from Electro-Nucleonics (Fairfield, NJ). Concanavalin A–Sepharose, heparin–Sepharose, and cyanogen bromide activated Sepharose were supplied by Pharmacia Fine Chemicals (Piscataway, NJ). Carboxymethyl-Bio-Gel-agarose was obtained from Bio-Rad (Richmond, CA), while the Ultrogel AcA 54 was obtained form LKB (Rockville, MD).

Interferon and Anti-interferon Monoclonal Antibodies. HuIFN-γ was produced as previously described (Braude, 1983a). Briefly, leukocytes from human buffy coats were induced with a combination of A-23187 (0.5 μg/mL) and

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[1] Abbreviations: IFN, interferon; HuIFN-α, human α-interferon; HuIFN-β, human β-interferon; HuIFN-γ, human γ-interferon; CPG, Controlled Pore Glass; Con A/S, concanavalin A–Sepharose; H/S, heparin–Sepharose; PBS, phosphate-buffered saline; PB, 20 mM phosphate buffer, pH 7.2; CM-BGA, carboxymethyl-Bio-Gel–agarose; α-MM, methyl α-D-mannopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton(s); IEP, isoelectric point; Tris, tris(hydroxymethyl)aminomethane; pfu, plaque-forming unit(s); TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

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