Interactions of Thrombospondin with Extracellular Matrix Proteins: Selective Binding to Type V Collagen

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ABSTRACT Thrombospondin (TS), a protein first described in platelets, was recently shown to be synthesized and secreted by endothelial cells, fibroblasts, and smooth muscle cells. The presence of TS in the extracellular matrix of cultured cells has prompted us to examine the associations of this protein with matrix macromolecules. Interactions of TS with both matrix and serum proteins were tested using an enzyme-linked immunosorbent assay. With this assay we assessed the binding of TS in solution to proteins adsorbed to polystyrene microtiter plates. Among collagens, platelet TS bound to type V but not to types I, III, or IV. This selective interaction was confirmed in experiments using proteins linked to cyanogen bromide-activated Sepharose. TS released from platelets in response to thrombin activation, as well as that secreted by endothelial cells in culture, bound to type V but not to type I collagen-Sepharose. No binding was observed to denatured type V collagen-Sepharose. The binding region for type V collagen was located in a chymotrypsin-produced fragment of TS with chains of M_r = 70,000, after reduction. Interactions of TS with a number of other proteins, including fibronectin, fibrinogen, and laminin, could be demonstrated using the enzyme-linked immunosorbent assay technique but the interpretation of these findings is difficult since comparable binding to protein-Sepharose was not always observed. Our findings suggest that both the extravascular distribution and function of TS in vivo may involve an interaction with type V collagen.

Thrombospondin (TS)[1] is a glycoprotein consisting of three, possibly identical, disulfide-bonded chains of ~140,000 mol wt (25, 31). The protein was initially described in platelets and was shown to be released from storage in α-granules by the action of thrombin (2, 18). A fraction of the secreted TS binds to the platelet surface (15, 39). Subsequently TS, or a protein very similar to it, was shown to be secreted by endothelial cells (9, 34, 36, 42, 45) and by various other cells in culture, including fibroblasts (21, 40), smooth muscle cells (40), and granular pneumocytes (43). The observation by immunofluorescence of TS in a fibrillar extracellular meshwork in cultured cells (21, 40) suggested that TS may function as a normal component of the extracellular matrix in vivo.

It has been proposed that TS acts as an endogenous lectin in platelet aggregation by binding to fibrinogen associated with the activated platelet surface (20). Support for this proposal has come from the observation that fibrinogen in solution can form a complex with TS adsorbed to a plastic surface (26). In addition, Lahav et al. (22) have provided evidence for the interaction of TS with fibronectin during platelet adhesion and aggregation. Using a radioactive cross-linking agent, an interaction was observed between TS released from activated platelets and fibronectin or collagen adsorbed to a glass surface (22).

In view of the synthesis of TS by a variety of cells and its location in the extracellular milieu, we thought it likely that its function in the extracellular matrix would involve interactions with matrix proteins. We therefore tested the interaction of TS with various purified extracellular matrix proteins, as well as with representative serum and intracellular proteins, by an enzyme-linked immunosorbent assay (ELISA) method. Further testing of positive interactions was performed by examination of the binding of ^{125}I-TS to proteins coupled to

[1] *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; TS, thrombospondin.

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Sepharose. We have observed selective binding of TS to type V collagen; this finding may provide a clue to the location and function of TS in the extracellular matrix.

MATERIALS AND METHODS

Preparation and Modification of Thrombospondin: TS was isolated from human platelets by the method of Lawler and Slayter (24) as modified by Raugi et al. (40), with the following changes. In some experiments the EDTA in the platelet suspension buffer was replaced with 1 mM $CaCl_2$. Activation of platelets was accomplished by adding human thrombin (obtained from W. Kisiel, University of Washington) to a final concentration of 0.7 National Institutes of Health U/ml and the thrombin was inactivated by addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. When calcium was present in the platelet suspension buffer, the platelets aggregated promptly after addition of thrombin. The platelet aggregate was rapidly removed by centrifugation at 900 *g* for 2 min at 4°C (J. Lawler, personal communication). The supernate was snap frozen and rewarmed at 37°C. Residual insoluble material was removed by centrifugation at 27,000 *g* for 20 min at 4°C (49). The protease inhibitor *N*-ethylmaleimide was added to the supernatant fraction to a final concentration of 1.25 mg/ml. The releasate was then applied to a Sepharose CL-4B column (2.5 × 100 cm), equilibrated in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, and either 2.5 mM EDTA or 1.0 mM $CaCl_2$, pH 7.5. The eluate was monitored by absorbance at 280 nm and fractions containing TS were pooled and passed over a column containing 5 ml of heparin-Sepharose 6B (Pharmacia, Inc., Piscataway, NJ). The column was washed with 20 mM phosphate buffer, pH 7.5, containing 150 mM NaCl, and either 25 μM EDTA or 50 μM $CaCl_2$. A further wash with the same buffer containing 300 mM NaCl was performed, before elution of TS with 20 mM phosphate buffer containing 600 mM NaCl and either 25 μM EDTA or 50 μM $CaCl_2$. Yields of up to 6 mg of TS were obtained from 4 U platelets, with a peak concentration of eluate from the heparin-Sepharose column of ~2 mg/ml. Bovine platelet TS was prepared by a previously published method (40).

TS was iodinated with the Iodo-Gen reagent (1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril; Pierce Chemical Co., Rockford, IL) using the method of Salacinski et al. (48). ^{125}I-TS was separated from free iodine by gel filtration on a column of G-25 (medium) Sephadex (Pharmacia, Inc.) equilibrated in PBS. Specific activities of 700–2,400 dpm/ng were obtained. After chromatography, BSA was added as a carrier to a final concentration of 1 mg/ml and the TS diluted (if necessary) to a maximum concentration of 100 μg/ml. Analysis showed >95% of the radioactivity to be precipitable in 10% trichloroacetic acid. SDS PAGE followed by autoradiography showed no discernible degradation from the iodination since all of the radioactivity was present in TS and in a small amount of a lower molecular weight derivative in the starting material.

Chymotryptic fragments of TS were prepared as described by Raugi et al. (Raugi, G. J., S. M. Mumby, and P. Bornstein, manuscript in preparation). Briefly, TS prepared in the presence of calcium was digested with α-chymotrypsin (three times crystallized, Worthington Diagnostics, Freehold, NJ) for 30 min at 37°C with an enzyme/substrate ratio of 1:200 (wt/wt). The reaction was terminated by adding diisopropylfluorophosphate (Aldrich Chemical Co., Milwaukee, WI) to a final concentration of 2 mM. Solutions of chymotryptic fragments were diluted fourfold to reduce the NaCl concentration to 150 mM before use for analysis of binding to ligand-Sepharose samples.

Other Proteins: Fibronectin was purified by gelatin-Sepharose chromatography of cryoprecipitated human plasma (10). BSA and human and sheep IgG were purchased from Miles Laboratories, Inc. (Elkhart, IN). Grade V chicken ovalbumin was purchased from Sigma Chemical Co. (St. Louis, MO). Bovine fibrinogen and human von Willebrand factor were provided by M. Chopek, University of Washington. Human coagulation factors IX_a and X_a were provided by W. Kisiel, University of Washington. Human plasma low density and high density lipoproteins were provided by J. Oram, University of Washington. Rabbit skeletal muscle phosphorylase b was obtained from E. H. Fischer, University of Washington. Catalase was purchased from Pharmacia Fine Chemicals (Div. of Pharmacia, Inc.). Lathyritic rat skin collagen (type I) was prepared as described by Bornstein and Piez (4). Human collagen types I, III, and IV were provided by Helene Sage, University of Washington. Human fetal membrane and bovine uterus type V collagens were obtained from Dietrich Hörlein, Bio-Gen, Geneva, and Beat Trüeb, University of Washington, respectively. A characterization of one of these preparations and other similarly prepared type V collagens has been reported (44). Denatured collagens were prepared by heating native collagen solutions at 55°C for 30 min.

Laminin was provided by A. Oohira (University of Washington) and had been fractionated from the culture medium of [^{3}H]proline-labeled PYS-2 cells by ammonium sulfate precipitation (37). The precipitate was dissolved in PBS containing 3 M urea and protease inhibitors (0.2 mM phenylmethanesulfonyl fluoride, 10 mM *N*-ethylmaleimide, and 2.5 mM EDTA). The proteins were resolved by DEAE cellulose (DE52, Whatman Laboratory Products, Inc.) (1) and hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) chromatography (34). Laminin purity was established both by Coomassie Blue staining and by fluorography of SDS polyacrylamide gels (37). Protein concentrations were determined by the method of Bradford (5) or Lowry et al. (28) using bovine pancreas α-chymotrypsin as a standard (Worthington Diagnostics).

ELISA: TS binding to proteins adsorbed to polystyrene was assayed by ELISA. Wells of Immulon 1 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 50 μl of proteins in solution at 5 μg/ml (in PBS; in some cases collagens were in 25 mM acetic acid). The microtiter plate was rinsed with 0.15 M NaCl in 0.05% Tween-20 before addition of TS. 50 μl of platelet TS at 5 μg/ml in a buffer containing 150 mM NaCl, 1.5 mM KH_2PO_4, 10.8 mM Na_2HPO_4, 2.7 mM KCl, 0.05% Tween-20, 0.02% NaN_3, and 1 mg/ml BSA (ELISA buffer) was incubated in the protein-coated wells for 1 h at 37°C. The plate was rinsed as before and 50 μl of rabbit anti-human platelet TS diluted 1:200 in ELISA buffer was incubated for 1 h at 37°C. The rabbit immunoglobulins had been enriched by repeated ammonium sulfate precipitation and antifibronectin activity had been removed by affinity chromatography on fibronectin-Sepharose (40). The microtiter plate was rinsed as before and incubated for 1 h at 37°C with alkaline phosphatase-conjugated goat anti-rabbit IgG (50 μl of 1:1,000 dilution). The plate was rinsed and then incubated with 50 μl of *p*-nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine, pH 9.8). The plate was incubated at room temperature until the most positively reacting wells reached an absorbance of 1.0–1.2 at 405 nm, as determined by a Dynatech microtiter plate autoreader (model MR580, Dynatech Laboratories). The data are reported after subtraction of a blank in which TS was omitted. With the exception of the assays for von Willebrand factor, the absorbance for the blanks were all very low (<10%) and did not vary significantly depending on the protein adsorbed to the plastic. The von Willebrand factor blank was <20% of the experimental value.

Binding of TS to Proteins Coupled to Sepharose: Proteins were coupled to cyanogen bromide-activated Sepharose (Pharmacia, Inc.) at 4°C following directions provided by the manufacturer. Briefly, proteins (2 mg/ml or 5 mg/ml) were solubilized into coupling buffer (0.1 M sodium bicarbonate, pH 8.3, containing 0.5 M NaCl). A protein solution twice the volume of the gel was rocked end-over-end overnight. The remaining reactive groups were blocked by mixing the gel with 0.2 M glycine, pH 8.0, overnight. Control gels were mixed first with bicarbonate buffer lacking protein and then with glycine buffer. The gels were rinsed extensively with the bicarbonate coupling buffer alternated with 0.1 M acetate buffer, pH 4, containing 0.5 M NaCl. The efficiency of coupling for native collagen types I and V and phosphorylase b was ~50%. The efficiency of coupling for denatured collagens was 85%. Fibrinogen, catalase, and fibronectin were coupled at >90% efficiency. The degree of coupling was determined by measuring protein concentration (28) of the coupling solution before and after the cross-linking reaction.

TS binding to ligand-Sepharose samples was assayed essentially as described by Lahav et al. (22). To a 0.1-ml sample of packed protein-coupled Sepharose was added either (*a*) 0.02 ml culture medium conditioned by endothelial cells; (*b*) 0.1 ml human platelet releasate (40); (*c*) 0.1 ml human platelet ^{125}I-TS at 10 μg/ml; (*d*) 0.25 ml purified human platelet TS (125 μg/ml); or (*e*) 0.25 ml of a chymotryptic digest of human platelet TS at ~250 μg/ml. The solutions were allowed to incubate with the Sepharose samples at room temperature for 15 min, with brief vortexing every 5 min. The bound and unbound fractions were then analyzed by SDS PAGE. Those ligand-Sepharose samples used for ^{125}I-TS binding were first rinsed with PBS containing 1 mg/ml BSA. After incubation with ^{125}I-TS, small equal aliquots of the bound and unbound fractions were analyzed by counting in a gamma counter (Beckman Instruments, Fullerton, CA, Gamma 4000) as well as by SDS PAGE.

Cell Culture: PYS-2 cells, from a mouse teratocarcinoma-derived cell line, were provided by J. Lehman, University of Colorado (Denver, CO). The cells were cultured and radiolabeled with [^{3}H]proline as described by Oohira et al. (37) for the isolation of laminin.

Bovine aortic endothelial cells were provided by S. Schwartz, University of Washington. The cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (50 μg/ml), and butyl *p*-hydroxybenzoate (0.4 μg/ml). Nearly confluent cultures were labeled overnight with 50 μCi/ml L-[^{35}S]methionine (1,244.5 Ci/mmol, New England Nuclear, Boston, MA) in medium containing methionine at only 10% of the normal level, and supplemented with 1 mg/ml BSA.

RESULTS

A number of proteins were tested for their ability to bind TS, as detected by a specific antiserum to TS in an ELISA. The assay has the advantage of using submicrogram quantities of

proteins and is easy to perform in an automated manner. The results of a large number of experiments that tested for the ability of TS to bind to proteins adsorbed to polystyrene are summarized in Table I. Similar results were obtained with human and bovine platelet TS. Purification of TS in the presence of Ca^{2+} is believed to preserve a more native structure of the protein (23). However, no differences were observed between the binding of human platelet TS purified in the presence of either 50 μM Ca^{2+} or EDTA (unpublished experiments).

A striking selective binding of TS to type V collagen was observed (Table I). However, no interaction between TS and types I, III, and IV collagens could be demonstrated. This distinction was not due to the lack of binding of the latter collagens to plastic since their presence was readily demonstrated by an ELISA using the appropriate specific antisera (data not shown). Collagens at 5 μg/ml, in PBS or 25 mM acetic acid, exhibited the same selective binding of TS to type V collagen. TS was also bound to heat-denatured type V collagen-coated wells. Prior digestion of type V collagen with highly purified bacterial collagenase abolished the binding of TS (data not shown).

TS also bound to the basement membrane protein, laminin. Among serum proteins, TS bound to fibronectin, fibrinogen, von Willebrand factor, factors IX_a and X_a and low density lipoprotein. TS did not bind to albumin, high density lipoprotein or IgG (Table I). The interaction with fibrinogen agrees with the finding reported by Leung and Nachman (26). In some instances quantitative differences in the extent of binding of TS may be due to differences in the amount of the protein ligand bound to plastic. However, the distinction between TS binding to low and high density lipoproteins cannot be attributed to lack of binding of the latter to plastic since specific antibodies were used in an ELISA to demonstrate adsorption of each lipoprotein (data not shown).

TABLE I

Binding of Purified Platelet Thrombospondin to Proteins Adsorbed to Polystyrene Microtiter Plates

Protein on polystyrene	Thrombospondin binding	
None	—	(20)
Matrix proteins		
Type I collagen, native	—	(25)
denatured	—	(2)
Type III collagen, native	—	(3)
Type IV collagen, native	—	(2)
Type V collagen, native	+++	(22)
denatured	+++	(4)
Laminin	++	(2)
Serum proteins		
Albumin	—	(6)
Plasma fibronectin	+	(9)
Fibrinogen	++	(29)
von Willebrand factor	++	(5)
Factor IX_a	+++	(2)
Factor X_a	+++	(2)
Low density lipoprotein	++	(4)
High density lipoprotein	—	(2)
IgG	—	(2)
Intracellular proteins		
Catalase	+++	(7)
Phosphorylase b	+++	(14)

The results, as assayed by the ELISA method, are reported in relative terms to summarize the results of many different experiments performed at different times. Each experiment included four or more of the proteins listed. No binding of TS above background is represented by —. Positive binding of TS is represented by +, ++, +++. +, 0.1–0.4 absorbance units at 405 nm, ++, 0.4–0.8, +++, 0.8–1.2. The number of determinations made on different days for each protein is given in parentheses. Each determination was made in duplicate or triplicate.

In view of the large number of positive interactions observed for TS with other proteins, we tested two cytoplasmic proteins, catalase and phosphorylase b, which we had expected to serve as negative controls. However, both of these proteins bound TS as extensively as did type V collagen (Table I). Some indication of the nature of the interaction of TS with proteins was obtained by altering the composition of the ELISA buffer. The effects of a higher concentration of Tween-20, high ionic strength, urea, and SDS on the binding of TS to several proteins are shown in Fig. 1. An increase in the concentration of Tween-20 to 1%, from the normal level of 0.05%, had no effect on TS binding (the extent of binding in lanes *1*, Fig. 1, were the same at both detergent concentrations). 1 M NaCl nearly abolished binding to type V collagen but had little effect on the binding of TS to fibrinogen or phosphorylase b. 4 M urea reduced binding to all three proteins but, again, the effect was most substantial for type V collagen. Finally, 0.1% SDS reduced the binding of TS to all the proteins tested. These findings suggest that the nature of the association of TS with type V collagen differs in its properties from the binding to fibrinogen or phosphorylase b.

To investigate further the specificity of binding, interactions of TS with proteins linked to Sepharose were tested (Table II). Iodinated TS was not bound significantly above background to either native or denatured type I collagen or denatured type V collagen. In contrast, highly significant binding to native type V collagen-Sepharose was observed. This binding was as extensive as that to heparin. Human platelet ^{125}I-TS bound equally well to type V collagen isolated from either human or bovine tissues (data not shown). Binding of TS to fibrinogen was modest but significant, and no binding to fibronectin above control levels was detected (Table II). It was of considerable interest that neither catalase nor phosphorylase b could bind TS significantly when these proteins were linked to Sepharose.

The selective binding of TS to type V collagen was demonstrated by incubating the complex mixture of proteins secreted by thrombin-activated platelets with type V collagen-Sepharose. In addition, control Sepharose, type I collagen-

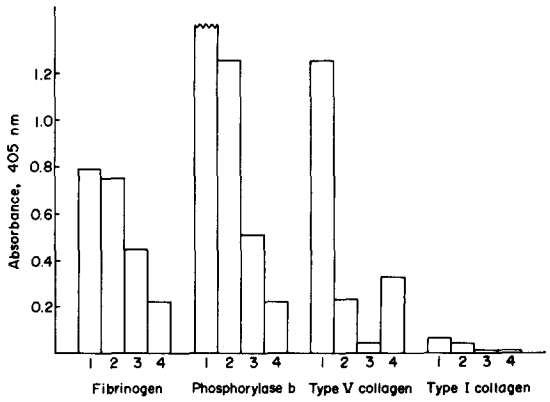

FIGURE 1 Effects of detergents, high ionic strength, and urea on the binding of thrombospondin to proteins adsorbed to polystyrene plates. Binding in the presence of 1% Tween-20 (lanes *1*), 1 M NaCl (lanes *2*), 4 M urea (lanes *3*) and 0.1% SDS (lanes *4*) was determined by the ELISA method. The levels of binding in 1% Tween-20 (lanes *1*) did not differ significantly from those found in the usual concentration of Tween-20, 0.05%.

TABLE II

Binding of ^{125}I-TS to Sepharose-coupled ligands

Ligand	[^{125}I]TS-bound
	%
Control	6 ± 2 (5)
Native type I collagen	6 ± 2 (6)
Denatured type I collagen	6 ± 2 (3)
Native type V collagen	65 ± 5 (8)*
Denatured type V collagen	6 ± 3 (3)
Fibrinogen	13 ± 2 (4)*
Plasma fibronectin	5 ± 1 (4)
Heparin	50 ± 13 (7)*
Catalase	10 ± 7 (4)
Phosphorylase b	6 ± 2 (4)

Iodinated platelet TS (1 μg, specific activity 700–2,400 dpm/ng, in PBS containing 1 mg/ml of BSA) was incubated for 15 min at 20°C with 100 μl of ligand-Sepharose beads. Beads were washed three times with PBS containing BSA, transferred to a clean tube with PBS, and boiled 3 min in SDS PAGE sample buffer to remove bound radioactivity. Results are expressed as counts per minute bound/counts per minute bound + counts per minute unbound. Values represent mean ± standard deviation. Values in parentheses indicate number of measurements.

* Value different from control: $p < 0.01$.

and heparin-Sepharose were used. The results, as analyzed by SDS PAGE, are shown in Fig. 2. Of the mixture of proteins released by platelets only TS was bound to a significant extent by type V-collagen Sepharose. TS, as well as other proteins, were bound to heparin-Sepharose whereas no platelet proteins were bound to type I collagen-Sepharose or control Sepharose. In the case of both type I and type V collagen-Sepharose, the hot SDS PAGE sample buffer used to elute proteins from the ligand-Sepharose also dissociated a fraction of type I and type V collagen, respectively (Fig. 2 *B*, lanes *2* and *3*). It should be noted that less of the TS in the platelet releasate bound to type V collagen than to heparin-Sepharose (Fig. 2) whereas iodinated TS bound equally well to the two ligands (Table II). It is possible that other proteins in the platelet releasate may have interfered with the ability of TS to bind to the type V collagen-Sepharose.

The selectivity of binding of TS to type V collagen was shown in yet another way by testing for the ability of type V collagen-Sepharose to bind TS secreted by endothelial cells into the culture medium. For comparison, control, ovalbumin, and type I collagen-derivatized Sepharose samples were used. Conditioned culture medium from endothelial cells labeled with [^{35}S]methionine was run over a denatured type I collagen-Sepharose column to remove fibronectin. The pretreated medium was incubated with the derivatized Sepharose samples and the interactions were analyzed by SDS PAGE and autoradiography. Binding of TS was detectable for type V collagen-Sepharose only (Fig. 3). Bands representing other proteins bound to type V collagen-Sepharose were also observed (lane *4*, Fig. 3 *B*). These proteins may be involved in the interaction of endothelial cell TS with type V collagen. However, a requirement for either of the two proteins is unlikely since purified platelet TS binds to type V collagen in the absence of any other proteins. Densitometric scanning of lanes *4* in Fig. 3, *A* and *B* indicated that 69% of the recovered TS in the conditioned medium was bound to type V collagen. If the culture medium had not been pretreated to remove fibronectin, <20% of the TS bound to type V collagen-Sepharose (data not shown). The total TS in the unbound and bound fractions from type V collagen-Sepharose (lanes *4*, Fig. 3) do not equal that in the starting material (lane *1*, Fig. 3 *A*). It is likely that some of the TS originally bound to type V collagen was lost during the washing of the Sepharose to remove nonspecifically-bound proteins.

In an attempt to locate the type V collagen-binding region

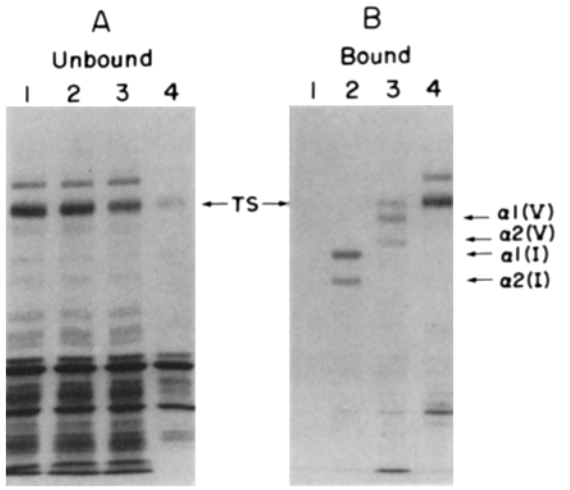

FIGURE 2 Binding of secreted platelet proteins to Sepharose-bound ligands. Proteins secreted by thrombin-activated platelets were incubated with samples of ligand-Sepharose and equal portions of the unbound and bound proteins were analyzed by SDS PAGE (6 and 10% composite gel) in the presence of dithiothreitol and Coomassie Blue staining. (*A*) Unbound platelet proteins; (*B*) proteins bound to ligand-Sepharose samples. Lanes *1*, control Sepharose; lanes *2*, type I collagen-Sepharose; lanes *3*, type V collagen-Sepharose; lanes *4*, heparin-Sepharose. Arrows indicate the positions of migration of thrombospondin (*TS*), type V collagen α-chains [*α1(V)*, *α2(V)*], and type I collagen α-chains [*α1(I)*, *α2(I)*]. Collagen chains were dissociated from the collagen-Sepharose samples by the hot SDS PAGE sample buffer used to elute bound proteins.

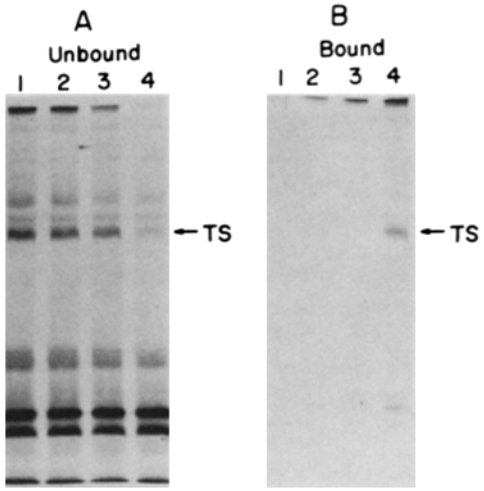

FIGURE 3 Ligand-Sepharose binding of endothelial cell secreted proteins after removal of fibronectin. Endothelial cell proteins were metabolically labeled with [^{35}S]methionine in the presence of 1 mg/ml BSA and were then passed over a denatured type I collagen-Sepharose column to remove fibronectin. Equal portions of conditioned medium proteins were then assayed with various ligand-Sepharose preparations. Unbound (A) and bound (*B*) samples were analyzed by SDS PAGE (6 and 10% composite gel) in the presence of dithiothreitol, and autoradiography. Lanes *1*, control-Sepharose; lanes *2*, ovalbumin-Sepharose; lanes *3*, type I collagen-Sepharose; lanes *4*, type V collagen-Sepharose. The arrow indicates the position of migration of thrombospondin (*TS*).

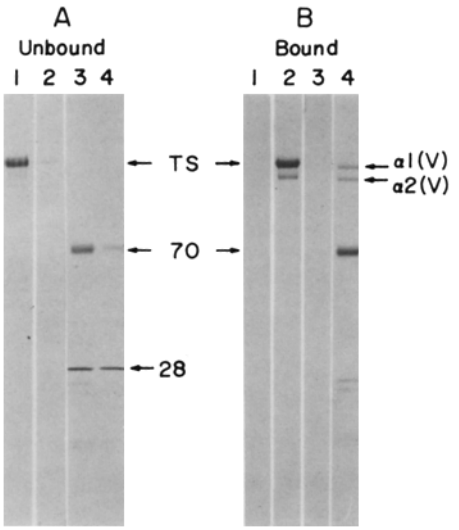

FIGURE 4 Binding of thrombospondin and its chymotryptic fragments to type V collagen-Sepharose. Unbound (*A*) and bound (*B*) fractions were resolved by SDS PAGE (4–20% gradient gel) in the presence of dithiothreitol and visualized by Coomassie Blue staining. Lanes *1*, TS and control Sepharose; lanes *2*, TS and type V collagen-Sepharose; lanes *3*, chymotryptic fragments and control Sepharose; lanes *4*, chymotryptic fragments and type V collagen-Sepharose. 12.5% of the unbound fractions and 24% of the bound fractions were loaded on the gel. The arrows indicate the positions of migration of thrombospondin (*TS*), the 70,000-mol-wt fragment (*70*), the 28,000-mol-wt fragment (*28*), and type V collagen α-chains [*α1(V)*, *α2(V)*].

in TS, the protein was cleaved with chymotrypsin under conditions that lead to the production of two stable fragments with chain molecular weights of 70,000 and 28,000 after reduction (Raugi, G. J., S. M. Mumby, and P. Bornstein, manuscript in preparation). The chain M_r = 28,000-fragment binds to heparin (Raugi, G. J., S. M. Mumby, and P. Bornstein, manuscript in preparation) and is presumably similar to the chain $M_r \simeq$ 30,000 heparin-binding fragment of TS described by others (8, 24). As shown in Fig. 4, purified TS bound almost quantitatively to type V collagen-Sepharose (lanes *2*) but not to control Sepharose (lanes *1*). When a chymotryptic digest of TS was incubated with type V collagen-Sepharose, selective binding of the larger fragment, composed of M_r = 70,000 chains, was observed; essentially no binding of the heparin-binding fragment occurred (lanes *4*). Control Sepharose bound neither of the fragments (lanes *3*). These experiments provide preliminary evidence for a domain structure with multiple binding sites for TS, analogous to that described for fibronectin (19).

DISCUSSION

The identification of TS in the extracellular matrix of cultured cells (21, 40) has led us to examine whether specific interactions with matrix macromolecules could be demonstrated. Such interactions might provide information regarding the distribution and function of TS in vivo. We initially chose the ELISA method to detect protein interactions because of its economy in use of protein, ease, and speed. A striking selectivity for binding of type V collagen, to the exclusion of types I, III, and IV, was observed (Table I). However, a perplexing number of positive reactions with other proteins was observed. In the cases of fibrinogen and fibronectin, interactions with TS had been reported under different experimental conditions (22, 26); however, with cytoplasmic proteins such as catalase and phosphorylase b, an association was not expected.

It seemed possible that the adsorption of proteins to a plastic surface might permit anomalous interactions. We attempted to inhibit such possibly adventitious interactions, unsuccessfully, by increasing the concentration of detergent during the TS binding phase of the ELISA assay (Fig. 1). Indeed, the interaction of phosphorylase b with TS withstood high ionic strength whereas that of type V collagen did not (Fig. 1). We therefore assessed the ability of a number of different proteins, coupled in solution to Sepharose, to bind TS. Selective binding of ^{125}I-TS to type V collagen was maintained under these conditions (Table II). Some indication of specificity was provided by the finding that binding was abolished when type V collagen was heat denatured prior to linkage to Sepharose. It therefore appears that the native structure of the triple helix is required for the complex formation between TS and type V collagen coupled to Sepharose.

Additional support for the interaction of TS and type V collagen came from the demonstration that TS could be selectively bound to type V collagen-Sepharose from a complex mixture of proteins present in the supernatant fraction of thrombin-activated platelets (Fig. 2) or in the conditioned medium of endothelial cells. In the latter case more extensive binding could be shown when fibronectin was removed prior to the interaction (Fig. 3). Increased interaction of TS with type V collagen caused by removal of fibronectin could be indicative of TS and fibronectin binding to the same site on the collagen molecule.

No interaction of platelet ^{125}I-TS or endothelial cell TS with catalase- or phosphorylase-Sepharose was observed (Table II and unpublished observations), although platelet TS did bind to the enzymes when they were adsorbed to plastic (Table I). The interaction of TS with the cytosolic enzymes was not expected and may not be physiologically relevant. We have also detected binding of fibrinogen, von Willebrand factor, and type V collagen to phosphorylase b adsorbed to plastic. Adsorption of the enzymes to plastic may cause a conformational change resulting in the exposure of nonspecific protein binding sites. It has been demonstrated that adsorption of fibronectin to hydrophobic polystyrene causes conformational changes in the fibronectin molecule as determined by assay of biological activity and reactivity with antifibronectin (16, 17). These findings, together with a recently published study of the binding of proteoglycan link protein to collagens (6), indicate that the ELISA technique may be useful as a screening procedure but that positive interactions should be confirmed by another binding assay.

The structural basis for the selective binding of TS to type V collagen, as opposed to other closely related collagens such as types I or III, is not known. However, type V collagen differs from other interstitial collagens in posessing (*a*) a markedly reduced susceptibility to vertebrate collagenase (44), (*b*) a higher proportion of larger hydrophobic amino acids (35), and (*c*) different fibril-forming capability (7). If a limited TS-binding domain can be defined in type V collagen, its structure would be of interest. An analysis of the binding of TS to type V procollagen, secreted by A204 rhabdomyosarcoma cells (1), would also be informative.

We have preliminary evidence that the region of TS responsible for binding to type V collagen differs in location from the heparin-binding site. As shown in Fig. 4, a large

chymotryptic fragment of TS with chains of $M_r = 70,000$ after reduction bound to type V collagen, whereas the smaller chymotryptic fragment did not. The $M_r = 28,000$ chymotryptic fragment binds to heparin (Raugi, G. J., S. M. Mumby, and P. Bornstein, manuscript in preparation) and is presumably similar to the heparin-binding fragment generated by thrombin, plasmin, trypsin, or thermolysin (8, 24). These findings suggest that thrombospondin may be another example of a modular protein, such as fibronectin, in which discrete, relatively protease-resistant binding domains are separated by more flexible protease-sensitive regions (19, 41).

Gartner et al. (12) have reported that an endogenous lectin, secreted by activated platelets, binds to fibrinogen on the platelet surface. It has been suggested by Jaffe et al. (20) that TS functions as such a lectin. In their view platelets interact with each other by intercellular bridges of multivalent TS anchored to fibrinogen. This model is supported by the recent demonstration that fibrinogen in solution binds to TS adsorbed to a plastic surface (26). In our experience the inverse reaction, i.e., binding of TS to adsorbed fibrinogen, also occurs (Table I). An interaction between ^{125}I-TS and fibrinogen-Sepharose could also be demonstrated, although the extent of the interaction was not as great as that between TS and type V collagen or heparin (Table II).

The interactions between TS and type V collagen or fibrinogen should be considered in the context of the interactions with other proteins that have been detected, using the modified ELISA technique alone. The interaction with von Willebrand factor may be physiologically significant in that the protein is thought to play a role in platelet aggregation and adhesion, particularly at sites of vascular injury (11, 47). It should be noted, however, that Leung and Nachman (26) were unable to demonstrate an interaction of fluid phase von Willebrand factor with adsorbed TS. The binding of TS to factors IX_a and X_a may be a consequence of the ability of these serine proteases to cleave TS (Raugi, G. J., S. M. Mumby, and P. Bornstein, manuscript in preparation). TS could also play a structural role in association with laminin in basement membranes. The reason for the binding of TS to low density lipoprotein, yet to be confirmed by other means, is currently not understood. It is, however, interesting that TS has been shown by immunofluorescence techniques to be located in some basement membranes and in the thickened intima of atherosclerotic aorta (Wight, T., G. J. Raugi, S. M. Mumby, and P. Bornstein, manuscript in preparation), regions in which association with laminin or low density lipoprotein respectively, would be possible.

A role for TS and fibronectin in platelet adhesion or aggregation has been suggested by Lahav et al. (22). Using a radioactive cross-linking agent, these workers showed an interaction of TS on the surface of platelets with fibronectin or type I collagen adsorbed to glass. Binding of endothelial cell TS to plasma fibronectin coupled to Sepharose was also demonstrated. We were unable to observe binding of endothelial cell TS to fibronectin-Sepharose, although fibronectin in the culture medium of endothelial cells bound to TS-Sepharose (unpublished data). A moderate interaction of platelet TS with fibronectin bound to plastic was also shown by the ELISA method (Table I). Although an interaction between type I collagen and TS on the platelet surface was observed by Lahav et al. (22), we did not detect binding of purified TS to type I collagen adsorbed to plastic (Table I) or coupled to Sepharose (Figs. 2 and 3). It is possible that other factors on the platelet surface are required for an interaction between the two proteins to occur.

The selective binding of TS to type V collagen raises the possibility that this interaction may provide a clue to the extravascular location and function of TS. Type V collagen has been variously described as existing in or adjacent to basement membranes (3, 29), in the pericellular matrix (13, 14), in the renal interstitium in close apposition to basement membranes (32), and in the extracellular matrices of dense connective tissues (27). Since both endothelial and smooth muscle cells synthesize type V collagen (30, 33, 46) it is also likely to exist in blood vessels. Parsons et al. (38) have recently shown that platelets adhere very poorly to type V collagen bound to plastic. The possibility exists that in vivo adhesion of platelets to a subendothelial matrix may be promoted by the presence of TS. The role that TS plays in connective tissues at a distance from blood vessels will require further study. We are currently examining the location of TS in tissues by immunofluorescence techniques in an attempt to answer such questions.

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Note Added In Proof: We have preliminary evidence that the ability of TS to bind to type V collagen may be enhanced by preparation of TS in the relatively low (≤ 50 μM) Ca^{2+} concentrations used in these experiments.

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