Interaction of Human Thrombospondin with Types I-V Collagen: Direct Binding and Electron Microscopy

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Abstract. Binding of thrombospondin (TSP) to types I-V collagen was examined by direct binding assays using ^{125}I-TSP and by visualization of rotary-shadowed intermolecular complexes in the electron microscope. The binding of TSP was highest to type V collagen in the absence of Ca, while lower but significant levels of binding were observed to all other collagen types in the presence or absence of Ca. Unlike intact TSP, the trimeric collagen-binding domain of TSP composed of 70-kD chains showed no Ca dependence in its binding to type V collagen. Further evidence for binding of TSP to types I and III collagen was obtained by competition studies in which these soluble collagens effectively inhibited binding of ^{125}I-TSP to immobilized type V collagen. The binding of TSP to type V collagen was inhibited by heparin and fucoidin, both high-affinity ligands of TSP's heparin-binding domain. mAb A6.1, which binds to the 70-kD domain of TSP, is also the best of a panel of anti-TSP mAbs at inhibiting the TSP-collagen interaction. Electron microscopy of rotary-shadowed replicas of TSP-collagen complexes revealed that all five types of collagen examined had a binding site for TSP at one end of the pepsinized, triple helical molecule. The specificity of this site was tested by examining the ability of BSA to form a complex with the end of the pepsinized collagens. Rotary-shadowed replicas revealed a low frequency of apparent BSA-collagen complexes, and histograms of these data showed no evidence for the preferential association of BSA with the end of the collagen molecules. In addition to the specific end site, type V collagen had an internal binding site for TSP located about two-thirds of the distance along the length of the collagen molecule from the end site. The internal binding site for TSP on type V collagen is apparently the site responsible for the higher affinity binding of TSP to that protein observed in direct binding assays. The trimeric 70-kD collagen-binding domain of TSP bound to the same sites on the collagens as did intact TSP.

THROMBOSPONDIN (TSP)[1] was first described as a glycoprotein released from alpha-granules of platelets upon stimulation with thrombin (Baenziger et al., 1971, 1972) and was later proposed as one of the factors involved in platelet aggregation (Jaffe et al., 1982). This functional role for TSP has been substantiated by recent reports that polyclonal antibodies against either intact TSP (Leung, 1984; Nurden et al., 1983) or the heparin-binding domain (Gartner et al., 1984) inhibit platelet aggregation and by the characterization of an mAb against TSP, which blocks aggregation of gel-filtered human platelets stimulated with thrombin or A23187 (Dixit et al., 1985a). We have previously described the ability of TSP to agglutinate trypsinized, fixed human erythrocytes (Haverstick et al., 1984; Dixit et al., 1985b) and fixed, activated platelets (Haverstick et al., 1985; Dixit et al., 1985b), and it has been shown that TSP released from platelets can bind to the activated platelet surface via an as yet unidentified receptor(s) (Phillips et al., 1980; Leung and Nachman, 1982; Roberts et al., 1985). Thus a role for TSP in the process of platelet aggregation has now been well established, although the precise nature of its participation in this complex reaction is not clear.

In the native state, TSP exists as a trimer of identical subunits of ~150,000-180,000 mol wt (depending on the SDS gel system) cross-linked by disulfide bonds (Baenziger et al., 1972; Lawler et al., 1978). Rotary-shadowed replicas of calcium-replete TSP viewed in the electron microscope have revealed that each subunit consists of a large globular region and a smaller globular region connected by a slender stalk (Galvin et al., 1985; Lawler et al., 1985). To form the native structure, three of these subunits are joined at a single point on the slender connecting arm near the small globular domain. The small globular region is the heparin-binding domain, which comprises the amino terminal of the protein chain (Galvin et al., 1985; Lawler et al., 1985). The stalk

region is thought to contain a binding site for type V collagen (Mumby et al., 1984), whereas the large carboxy-terminal globular domain includes binding sites for fibrinogen, Ca, and perhaps other as yet unidentified receptors (Dixit et al., 1984a, 1986a). When calcium is removed from the native molecule by chelation with EDTA or EGTA, the large globular domain appears smaller in diameter and the connecting arm longer (Dixit et al., 1986a; Lawler et al., 1985) as if the large domain has partially "unraveled." We have characterized mAbs that bind weakly or not at all to calcium-replete TSP but have high affinity for EDTA-treated TSP, indicating that removal of calcium exposes regions of the molecule otherwise unavailable for interaction. Both proteolysis and binding studies indicate that the conformational changes brought about in TSP by the removal of Ca are fully reversible when Ca is restored (Lawler et al., 1985; Dixit et al., 1986a). Electron microscopic localization of the epitopes for these calcium-sensitive mAbs have shown them to be on the connecting arm just where it enters the large globular domain or in the remnant of the large globular domain itself (Dixit et al., 1986a). These epitopes are in precisely those regions seen to "unravel" upon removal of Ca from TSP (Dixit et al., 1986a; Lawler et al., 1985).

In addition to being present in platelets, TSP is synthesized and secreted by a wide variety of cell types in culture including endothelial cells (Doyle et al., 1980; McPherson et al., 1981; Mosher et al., 1982; Sage et al., 1981), fibroblasts (Jaffe et al., 1983; Raugi et al., 1982), smooth muscle cells (Raugi et al., 1982), granular pneumocytes (Sage et al., 1983), macrophages (Jaffe et al., 1985), glial cells (Asch et al., 1986), and keratinocytes (Wikner et al., 1987). Immunofluorescent localization of TSP has shown that the protein is present in basement membrane regions beneath glandular epithelium in skin and lung, at the dermal-epidermal junction in skin, in interstitial areas of skeletal muscle, and in luminal portions of large blood vessels (Wight et al., 1985). Localization of TSP in cultured cells found it to be incorporated into the extracellular matrix of aortic endothelial cells, human fibroblasts, and human aortic smooth muscle cells (Raugi et al., 1982; Jaffe et al., 1983). These findings suggest that, in addition to its role in platelet aggregation, TSP is a more general component of the extracellular matrix. In view of this possibility, Mumby et al. (1984) examined the interaction of TSP with a number of extracellular matrix proteins, in particular collagen types I-V. Using a semiquantitative enzyme-linked immunosorbent assay as well as binding of iodinated TSP to collagen-Sepharose, they found a significant interaction between TSP and type V collagen but could detect no interaction with types I, III, or IV. We have examined the interaction of TSP with types I-V collagen in more detail using both a quantitative direct binding assay and electron microscopic observations of rotary-shadowed replicas of TSP-collagen complexes. We have found that TSP interacts specifically with all collagen types examined, but that type V collagen contains an additional binding site of apparently higher affinity for TSP.

Materials and Methods

Protein Purification

Calcium-replete human TSP was purified from the supernatants of thrombin-stimulated platelets as previously described (Dixit et al., 1984b). Types

I-V collagens were obtained from Dr. Robert Burgeson (Shriner's Hospital, Portland, OR), type III collagen from Dr. John Jeffrey (Washington University School of Medicine, St. Louis, MO), and type V collagen from Dr. Edmond Crouch (Jewish Hospital, St. Louis, MO). The collagens were prepared by limited pepsin digestion and differential salt precipitation (Trelstad et al., 1976; Burgeson et al., 1976; Burgeson and Hollister, 1979; Crouch et al., 1980). To prepare the TSP collagen-binding domain, TSP in Tris-buffered saline (TBS; 0.02 M Tris, 0.15 M NaCl, pH 7.6) with 5 mM EDTA added was digested with chymotrypsin as described (Dixit et al., 1986a). The digest was applied to a Sephadex G-100 column equilibrated with TBS, and the trimer of 70-kD chains was eluted at the void volume.

Iodinations

TSP and the 70-kD fragment of TSP were iodinated using Na-^{125}I and Iodo-Beads (Pierce Chemical Co., Rockford, IL) and purified by gel filtration on a column of G-25 Sephadex (Sigma Chemical Co., St. Louis, MO). Incorporation of ^{125}I ranged from 30 to 40%, and specific activities of 1–2 × 10^6 cpm/µg were obtained. The purity of the iodinated products was analyzed by TCA precipitation (with 95% of the ^{125}I being protein bound) and by SDS PAGE followed by autoradiography, which showed that all of the radioactivity was associated with a band corresponding to the appropriate protein.

Direct Binding Assay

Native collagen (type I, II, III, IV, or V), 50 µl at 50 µg/ml in either TBS with 5 mM calcium chloride or 5 mM EDTA, was placed in each of the wells of a 96-well polystyrene Removawell palte (Dynatech Laboratories, Inc., Alexandria, VA) and incubated for 2 h at 37°C or overnight at 4°C to coat the wells with collagen. Remaining protein-binding sites were then blocked by incubating the wells with 50 µl of BSA (1% in TBS with 5 mM calcium chloride or 5 mM EDTA) for 30 min at room temperature followed by addition of ^{125}I-TSP (1–2 × 10^5 cpm/well) in TBS with 1% BSA and 5 mM calcium chloride or 5 mM EDTA for 3–4 h at room temperature or overnight at 4°C. Preliminary studies showed that either condition assured that equilibrium was attained. The plates were then washed five times with TBS with calcium chloride or EDTA, and each well was counted in a gamma-counter. For examining the Ca-dependence of the type V collagen–TSP interaction, the free Ca concentration was varied as indicated by combining various concentrations of calcium chloride and EGTA. For inhibition studies, the indicated concentrations of soluble type I, III, or V collagen were included with ^{125}I-TSP during the binding step.

To determine if the different collagen types bound to the plastic wells with similar efficiencies, the collagens were coated on wells as above. Instead of blocking the wells with BSA, the wells were washed with TBS and then water. The collagen bound to triplicate sets of wells was determined by hydrolysis (with 6 N HCl for 24 h at 110°C under reduced pressure) and subsequent amino acid analysis. This was performed on HPLC equipment (Waters Instruments, Inc., Rochester, MN) using a sodium ion-exchange column and buffers from Pickering Laboratories (Mountain View, CA). Proline and hydroxyproline were detected by post column reaction with o-phthaldehyde after hypochlorite oxidation and were used to calculate the amount of each collagen type bound. The detection limit of this system is 50 pmol per amino acid. Analyses were performed by the Washington University Protein Chemistry Laboratory, Dr. G. A. Grant, Director. These experiments revealed that types I, III, and V collagens bound to the plastic wells with identical efficiencies (<5% variation). Thus any differences in subsequent TSP binding experiments represent real differences in the amount of TSP bound rather that differences in the amounts of collagen on the wells available for binding of TSP. It should be noted that we observed dramatic differences between the binding efficiencies of pepsinized and unpepsinized collagens in these callibration experiments. The unpepsinized collagens bound to the plastic wells with a much lower efficiency. Thus TSP binding experiments were only performed using immobilized pepsinized collagen types.

Electron Microscopy

For visualization by electron microscopy, all the collagens were dialyzed versus 0.2 M ammonium bicarbonate, diluted to 200 µg/ml, and brought to 50% with glycerol. The solution was then sprayed onto freshly cleaved chips of mica and rotary-shadowed with tungsten followed by carbon coating as described elsewhere (Galvin et al., 1985). The replicas were floated off onto distilled water and picked up on 300-mesh copper grids for viewing in a Philips 201 transmission electron microscope. To examine the interaction between TSP and collagen, TSP or its purified collagen-binding domain (200 µl at 200 µg/ml) in TBS with 2 mM calcium chloride or with 2 mM

Table I. Binding of ^{125}I-TSP to Immobilized Collagens in the Presence of 5 mM EDTA

Immobilized protein	cpm bound ± SD
Type I collagen	5,323 ± 399*
Type II collagen	5,324 ± 1,297‡
Type III collagen	4,941 ± 433*
Type IV collagen	2,631 ± 352§
Type V collagen	25,397 ± 1,266*
Type V collagen in 2 mM Ca	5,535 ± 367*
BSA	1,781 ± 231

* $P < 0.001$.
‡ $P < 0.005$
§ $P < 0.025$.

EGTA was added to 200 µl of collagen (type I, III, IV, or V) at 200 µg/ml and incubated for 1–2 h at room temperature. The solution was then brought to 50% with glycerol, sprayed onto mica, and rotary-replicated as described above. Micrographs of the collagens and TSP–collagen complexes were printed at a nominal magnification of 88,000, and the lengths of collagen molecules were measured with an electronic graphics digitizer (model 1224; Numonics Corp., Lansdale, PA). We used the criteria applied by Charonis et al. (1985) in analyzing the data, except we scored all TSP molecules in contact with a collagen molecule, including those that were found crossing over collagens. Some of these TSP molecules may have settled onto the collagen during the drying of the grids rather than associating specifically with collagen. These events would be expected to occur at random and at worst would contribute to the background scatter in the histograms of these data.

Results

Direct Binding Assay

In their report of the binding of TSP to type V collagen, Mumby et al. (1984) noted that the ability of TSP to bind collagen might be enhanced at low Ca concentrations. We therefore assayed the binding of our iodinated TSP preparations to the various collagen types in the presence of 2 mM Ca and 5 mM EDTA or EGTA. When Ca was present, binding to all the collagen types was low; however, addition of 5 mM EDTA or EGTA to the incubations produced highly repeatable levels of binding that were significantly above back-
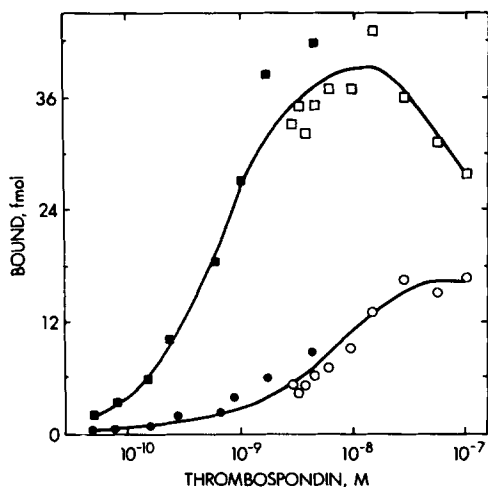

Figure 1. Binding of ^{125}I-TSP to type V collagen as a function of TSP concentration. *Circles*, binding in the presence of 5 mM Ca; *squares*, in the presence of 5 mM EDTA. Each type of symbol represents independent experiments.
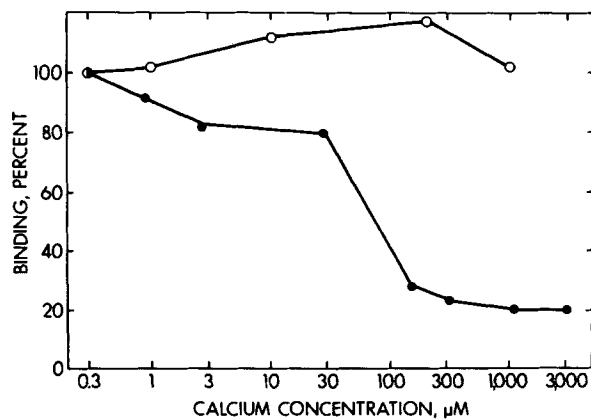

Figure 2. Binding of ^{125}I-TSP and ^{125}I-70-kD chymotryptic fragment of TSP to type V collagen determined under conditions of varying Ca concentration. *Solid circles*, TSP binding; and *open circles*, 70-kD binding.

ground. Table I lists the amounts of ^{125}I-TSP bound to collagen types I–V in the presence of 5 mM EDTA. The values given here represent means from two separate well plate assays with triplicate samples within each experiment. As shown in the table, control wells were coated with BSA. The data in Table I have not been corrected for the level of binding to BSA. Binding of TSP to all five types of collagen was significantly above the BSA control when tested by a t test; however, binding to type V was the most impressive, nearly 10 times higher than binding to any of the other collagens. However, even type V collagen in the presence of Ca (Table I) gave a level of binding comparable to the other collagen types in the absence of Ca.

The difference in the apparent affinity of ^{125}I-TSP for type V collagen in the presence and absence of Ca is shown in Fig. 1 where the amount of TSP bound is plotted as a function of increasing TSP concentration. The slope of the linear portion of the curve is steeper in the presence of EDTA, and the final asymptotic level of the curve is higher. This figure shows that the primary effect of Ca chelation is to simply shift the TSP binding curve to lower concentrations, thus causing an apparent increase in the affinity of the binding interaction. The effects seen in Table I appear to be more pronounced because those binding experiments were conducted at low TSP concentrations (<1 nM). The decrease in the binding of TSP at high concentrations in the absence of Ca is probably due to the trimeric and hence multivalent nature of TSP. Multivalent ligands such as lectins often exhibit this strong apparent negative cooperativity at higher concentrations (Bartles and Frazier, 1982). A more detailed analysis of the effect of Ca concentration on TSP binding to type V collagen was done by precisely varying the free Ca concentration in the incubation buffers using combinations of calcium chloride and EGTA (Ca buffers). The results of this experiment are shown in Fig. 2 where binding of ^{125}I-TSP is represented by the solid circles. A decrease in the amount of TSP binding to type V collagen was observed at Ca concentrations above 30 µM. This transitional point is similar to the level of free Ca previously estimated for the conformational change in TSP, which occurs upon Ca removal as measured by binding of an mAb A6.1, which recognizes a Ca-sensitive epitope in TSP (Dixit et al., 1986a). These results imply that the portion of the TSP molecule that binds to type

V collagen is near the region affected by this Ca-dependent conformational change. Interestingly, the epitope for mAb A6.1 is contained within the 70-kD chymotryptic fragment, which Mumby et al. (1984) reported to contain the binding site involved in the TSP-type V collagen interaction. Since both the small and large globular domains have been removed from this fragment, leaving essentially the long connecting arms of the molecule (Dixit et al., 1986a, Lawler et al., 1985), we examined the binding of the trimer composed of the 70-kD chymotryptic fragments to type V collagen as a function of free Ca concentration (Fig. 2, open circles). The amount of binding of the fragment was adjusted to be the same as that of ^{125}I-TSP in the absence of Ca. Increasing Ca concentration left the binding of the trimer of 70-kD fragments unimpaired, in marked contrast to the reduction in binding of intact TSP. Thus the binding of the 70-kD fragment to type V collagen appears to be completely independent of Ca, confirming that the conformational constraints on the collagen binding site(s) have been removed. These data, along with those in Fig. 1, reinforce the idea that the collagen binding structure of TSP is not inherently inhibited by Ca, but exists in a part of the molecule that is partially masked by the binding of Ca to other parts of TSP.

Although statistically significant, the binding of ^{125}I-TSP to types I and III collagens was very low and thus difficult to analyze with the direct binding assay (Table I). Interactions of these collagens with TSP were studied by examining their ability to compete with TSP for binding to type V collagen immobilized on a surface. The results of these competition studies are shown in Fig. 3. Soluble type III collagen was as effective as type V in completely blocking binding of TSP to type V collagen-coated polystyrene wells. A higher concentration of soluble type I collagen was required to achieve detectable competition, and complete competition with this collagen was not achieved at the concentrations used in this assay. The ability of soluble types I and III collagen to interfere with the interaction of TSP with immobilized type V collagen substantiated the direct binding data, which had indicated a low but significant level of binding of TSP to these collagens. It should be noted that this competitive assay directly measures the ratio of affinities of TSP for type V
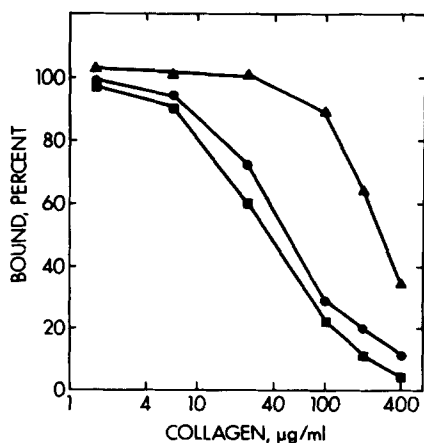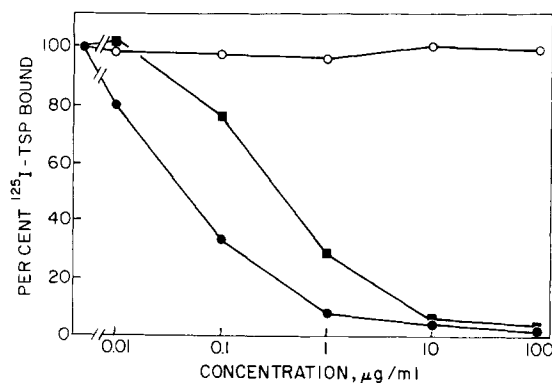

Figure 4. Effect of heparin (solid circles), fucoidin (squares), and hyaluronic acid (open circles) on the binding of ^{125}I-TSP to immobilized type V collagen. 100% binding in these experiments represents 55,000 cpm bound.

versus the competitor collagen type in the absence of such perturbations as washing of the wells. As such, these results indicate that even though the affinity of TSP for type III collagen may be rather substantial, the kinetics of dissociation of this complex are probably faster than the TSP-type V collagen complex, which is stable to washing.

Specificity of TSP-Collagen Binding

While the data above suggest that both TSP and its 70-kD chymotryptic fragment bind to type V collagen with reasonably high affinity, we sought other tests for the specificity of this interaction. Previous work has shown that TSP contains specific, high affinity binding sites for the glycosaminoglycan heparin. These sites are associated with the small amino-terminal globular domain, which has thus been termed the heparin-binding domain of TSP. The highly sulfated polysaccharide fucoidin has an affinity nearly as high as that of heparin for these sites, while other glycosaminoglycans have very low affinity. As shown in Fig. 4, heparin and fucoidin inhibit the binding of TSP to type V collagen, whereas hyaluronic acid does not. Furthermore, fibrinogen and fibronectin, both of which bind to TSP, do not inhibit the TSP-collagen interaction at concentrations up to 100 μg/ml (not shown). This suggests that other regions of the TSP molecule are involved in these interactions. We also tested our battery of anti-TSP mAbs (Dixit et al., 1985a, b, 1986a) as potential inhibitors of the TSP-type V collagen interaction. mAb A6.1, which we have previously shown to bind to the 70-kD chymotryptic fragment of TSP (Dixit et al., 1986a), was the most potent inhibitor, giving 50% inhibition of TSP binding at 1 μg/ml in the standard binding assay with immobilized type V collagen. Other mAbs that recognize other regions of TSP were from 5- to 200-fold less potent inhibitors of the TSP collagen interaction.

Electron Microscopy

The binding of TSP to types I, III, IV, and V collagen was further examined by direct observation in the electron microscope of rotary-shadowed replicas of the complexes formed between TSP and each of these collagen types. All four types of pepsinized collagen were initially characterized by SDS PAGE and by electron microscopy of rotary-shadowed replicas for comparison with previously published charac-

Figure 3. Competition of ^{125}I-TSP binding to immobilized type V collagen by soluble types I (triangles), III (circles), and V (squares) collagen. The amount bound is expressed as a function of increasing concentration of the soluble collagen.
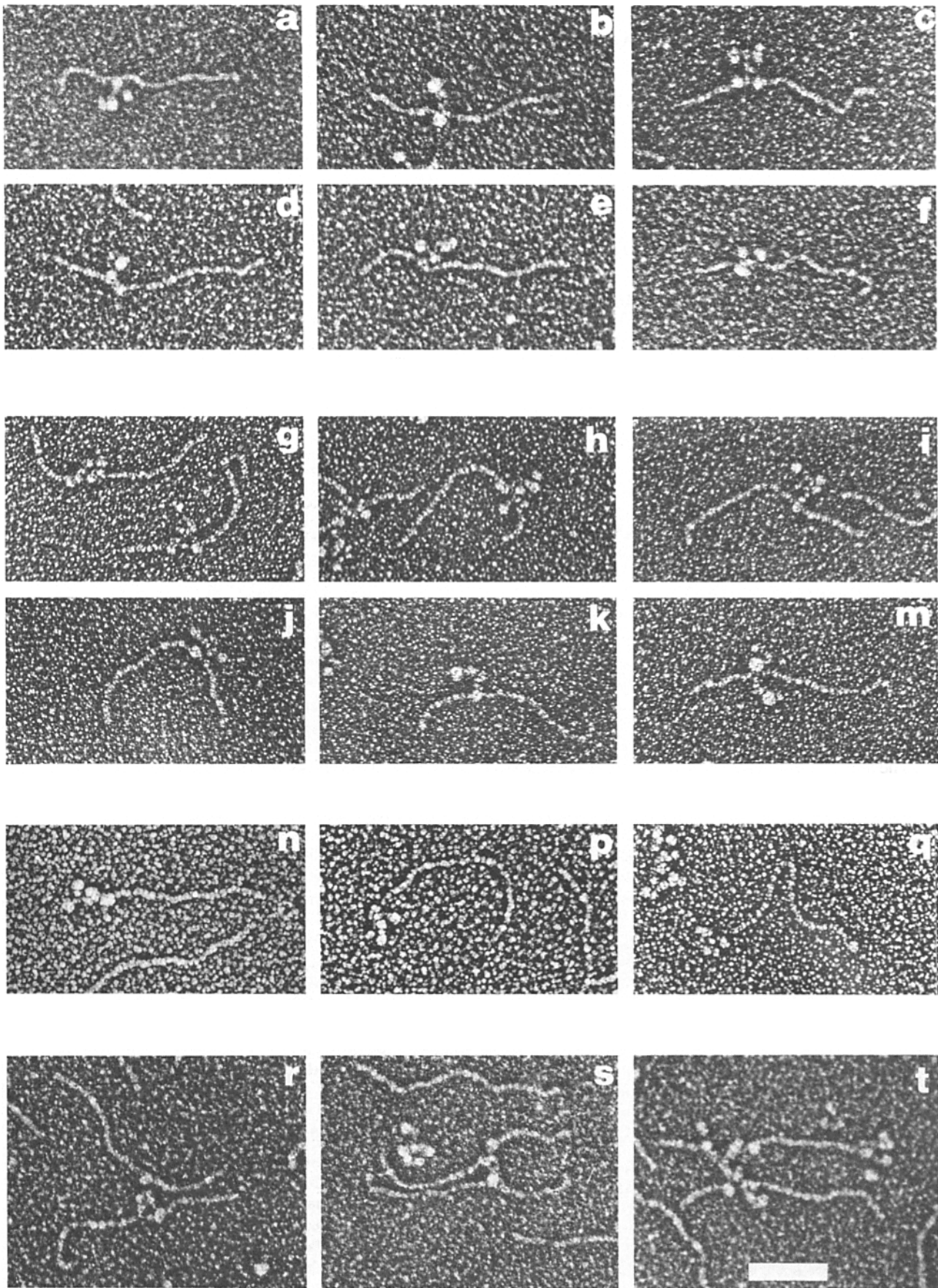
Figure 5. Electron micrographs of rotary-shadowed replicas of TSP bound to collagen molecules. (*a*−*f* and *r*−*t*) TSP in 2 mM Ca and type V collagen; (*g*−*m*) TSP in 2 mM EGTA and type V collagen; (*n*) TSP in 2 mM EGTA and type I collagen; (*p*) TSP in 2 mM EGTA and type III collagen; (*q*) TSP in 2 mM EGTA and type IV collagen. Bar, 100 nm.

terizations (Miller, 1984; Timpl and Martin, 1982; Timpl et al., 1981; Mayne et al., 1984; Fessler et al., 1981). For all collagen types, the characteristic bands were seen on SDS gels (data not shown). The native, monomeric collagen molecules were essentially indistinguishable from one another in the electron microscope and appeared as long flexible rods with a median length of 300 nm for types I, III, and V. Type IV collagen had more variability in length, ~350–400-nm long. Frequently, a hook-like structure was observed at one end of the collagen molecules (see views presented in Fig. 5).

Images of the complexes obtained when TSP and type I, III, IV, or V collagen were incubated together are shown in Fig. 5. The characteristic trimeric structure of the TSP molecule could be easily identified binding to the simple, rod-like structure of collagen. Sometimes the large globular domain of TSP seemed to be touching the collagen (Fig. 5, a, c, g, and i–k) and in other views, the small globular domains appeared to be involved (Fig. 5 e), but most often the connecting strands of the TSP trimer seemed to fall over the collagen molecule or even to be wrapped around it (Fig. 5, b, d, f, h, m, and r–t). Occasionally, a TSP molecule was seen forming a cross-link or bridge between two collagen molecules (Fig. 5, r–t).

To determine if the location on the collagen molecules where TSP appeared to bind was a specific binding site, we applied the analysis used by Furthmayr's group in the analysis of the interaction between laminin and type IV collagen (Charonis et al., 1985). This latter case is particularly interesting in that it was impossible to obtain any evidence of the specific binding of laminin to type IV collagen with direct binding assays. However, Charonis et al. (1985) argued that

Figure 7. Frequency histogram of distribution of BSA on type V collagen. Axes as in Fig. 6. The number of molecular complexes measured was 198.

clustering of laminin at a particular site on the type IV rod rather than randomly along it indicates a site with specificity. Thus we measured the distance from one end of the collagen rod to the point of TSP contact and expressed this as a fraction of the total length of the collagen molecule. Since we could not distinguish the carboxy termini from the amino termini of the pepsinized collagens used here, we calculated all ratios by measuring from the end closest to the attached TSP. Therefore, our ratios ranged from 0 to 0.5. Data were collected from TSP-collagen complexes formed either in the presence of 2 mM calcium chloride or 2 mM EGTA. The ratios obtained are plotted as frequency histograms in Fig. 6. The frequency distributions for TSP localized on types I, III, and IV collagen (Fig. 6, A–C) indicated that there was preferential binding of TSP to a site located at or very near one end of the pepsinized, triple helical collagen molecules. There were no differences between the distributions obtained for Ca-replete versus EGTA-treated samples.

A potential problem with the pepsinized collagens used in these experiments is that the end of the rod clipped by pepsin might provide a "sticky end," which would bind any protein at this site. To determine if this might be the case, we examined replicas of BSA mixed with each of the pepsinized collagen types, and collected data to generate the frequency distribution of the nearly spherical BSA molecule along the length of the collagen molecule. As shown for a mixture of BSA and type V collagen in Fig. 7, an essentially random histogram was obtained, indicating no preferential binding to the ends of the pepsinized collagen.

The frequency distribution of TSP along the length of the type V collagen monomer differed from that of the other collagens examined. While there was a preferred binding site at one end of the collagen molecule, there was, in addition, an internal binding site that had a peak frequency about one-third of the distance from one end. This internal binding site could be responsible for the high affinity or slowly dissociable binding of TSP to type IV collagen detected in our well plate assay and by Mumby et al. (1984). This idea is supported by the fact that binding to this internal site occurs with a frequency three to four times higher than binding to the end site (Fig. 6). As with the other collagens, the presence or absence of Ca did not alter the position of TSP binding sites on the type V molecule. However, by counting the number of collagen molecules with and without bound TSP, we found that 62% of the type V collagens in the EGTA-treated condition had at least one bound TSP, whereas 55% fell in that category for Ca-replete samples. This result would appear to
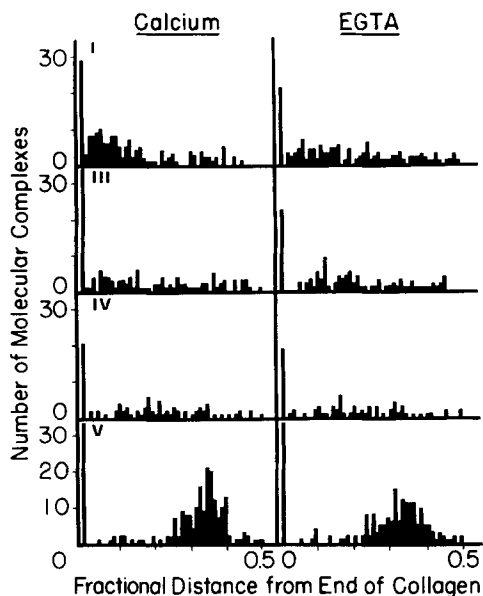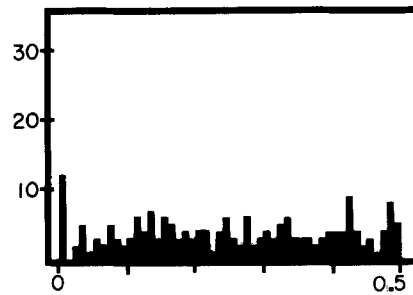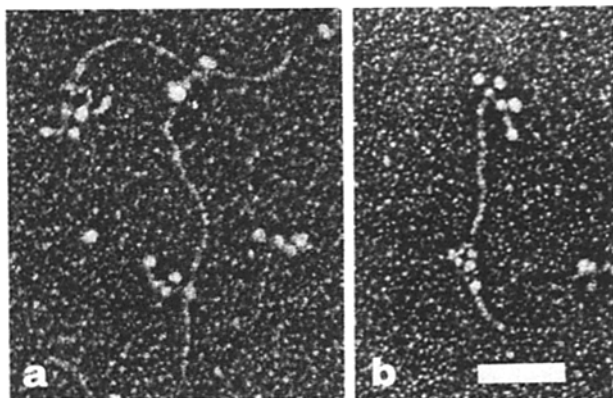

Figure 6. Frequency histograms of distributions of TSP molecules on types I, III, IV, and V collagen in 2 mM Ca or 2 mM EGTA. The vertical axis represents the number of molecular complexes measured, and the horizontal, the fractional distance from the nearest end of the collagen molecule to the bound TSP. The numbers of molecular complexes measured for each case were: In Ca, type I, 167; type III, 132; type IV, 90; type V, 221; in EGTA, type I, 131; type III, 107; type IV, 75; type V, 200.

conflict with the direct binding assays in which a larger difference in binding was found in the presence and absence of Ca (Table I). However, the higher concentrations of TSP and collagens used for microscopy lie in a region of the binding curve shown in Fig. 1, in which substantial binding would be detected even in the presence of Ca. The difference in the amount of binding observed with the two methods is further augmented by the fact that preparation of the samples for electron microscopy requires no dilution or perturbation of the binding equilibria, thus in principle allowing the detection of much lower affinity binding events than a binding assay in which dilution or washing steps are required.

While collecting data for the distribution of TSP binding sites along the type V triple helix, we found a number of type V collagen molecules that had two TSP molecules bound, one at the end site and one at the internal site. Ratio measurements from these "double labeled" collagen molecules were determined using the end binding site as the point from which the distance to the other binding site was measured. These data are displayed as a frequency distribution over the entire length of the type V collagen molecule shown in the histogram of Fig. 8. The internal binding site was most often located at a position about two-thirds of the length of the collagen molecule from the end binding site for TSP. Representative electron micrographs depicting this relationship between the two TSP binding sites on double-labeled collagens are shown in Fig. 8, a and b. In Fig. 8 a the TSP at the end of one collagen has cross-linked that molecule to another via the internal site on the neighboring collagen molecule. The finding that the internal site is located at a unique position relative to the end site also argues that the end site is unique, occurring at only one end of the collagen molecule.

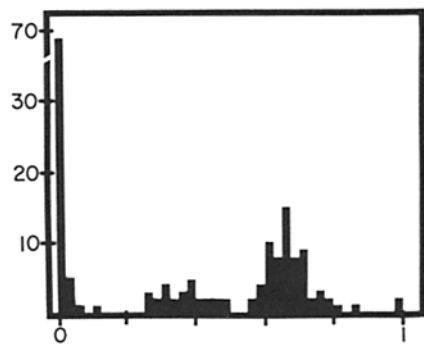To further investigate the specificity of the TSP–collagen

Figure 8. Frequency histogram of distribution of TSP molecules on type V collagen for examples in which each collagen molecule had one TSP bound at an end and another at an internal binding site. Representative electron micrographs are shown in *a* and *b*. Axes as in Fig. 6. The number of molecular complexes measured was 150. Bar, 100 nm.
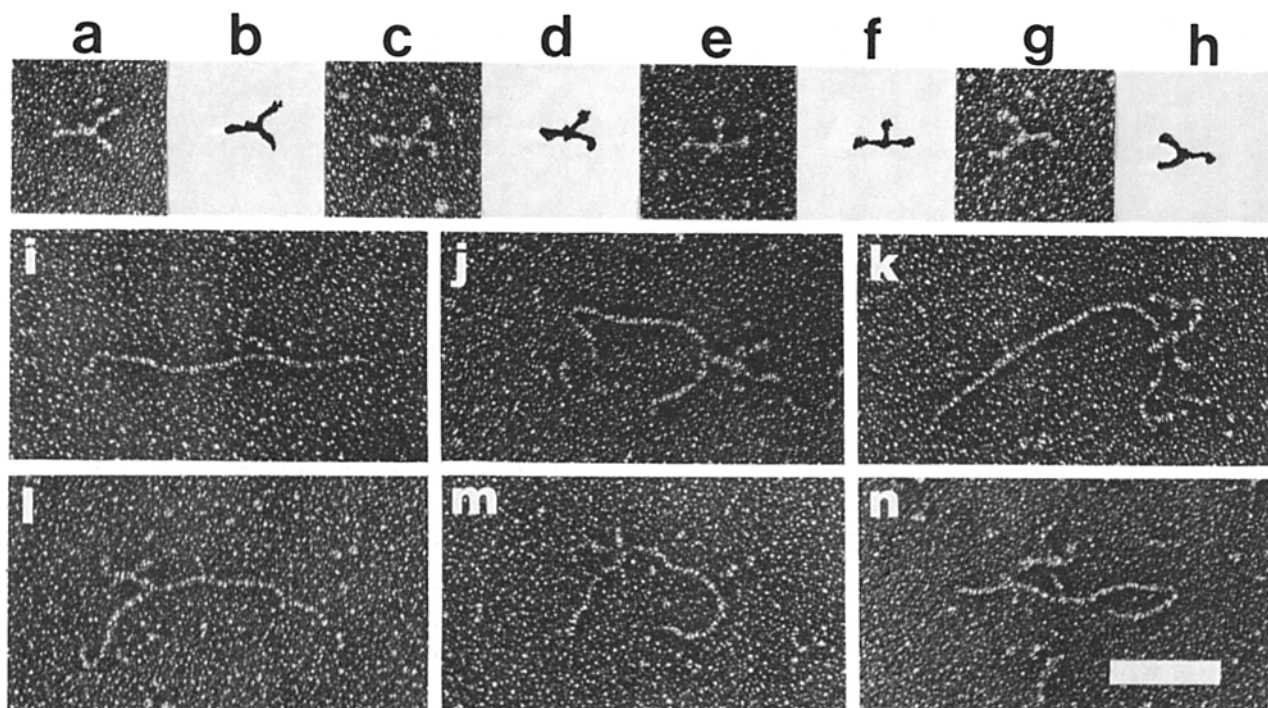

Figure 9. Electron micrographs of rotary-shadowed replicas of the trimeric TSP collagen-binding domain (*a*, *c*, *e*, and *g*) and of this collagen binding domain associated with the internal site on type V collagen (*i–n*). *b*, *d*, *f*, and *h* are ink drawings to aid in interpreting the panels to their left. Bar, 100 nm.
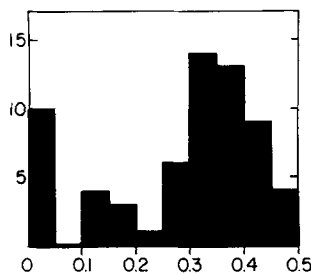
Figure 10. Frequency histogram of the distribution of the trimer of 70-kD chains on type V collagens. The vertical axis is the number of molecules measured and the horizontal the fractional distance from the nearest end of the collagen molecule to the bound TSP fragment. The number of molecular complexes measured was 65.

interaction, we examined replicas of the trimer of 70-kD TSP fragments and type V collagen. Fig. 9, *a*, *c*, *e*, and *g* shows representative images of the trimeric 70-kD chymotryptic fragment of TSP, and *b*, *d*, *f*, and *h* are drawings of the image to the left. The length of the residual arms of these trimers is 30.7 ± 7.4 (*n* = 573). This value is intermediate between the length of the connecting arms of intact TSP seen in the presence of Ca and its absence (26 and 43 nm, respectively) (Dixit et al., 1986*a*), suggesting that part of the extended or unraveled arm is cleaved by chymotrypsin. Alternatively, the slight bulge or globule often seen at the ends of the cleaved arms may mean that after cleavage, the ends of the arms fold up to some extent. Fig. 9, *i–n* shows examples of the trimeric collagen-binding domain interacting with the internal site on pepsinized type V collagen molecules. In addition, the fragment was seen associated with the end of the collagen rod in the case of types I, III, and V collagens. The histogram of these data for type V collagen is shown in Fig. 10. Thus it appears that the trimer composed of 70-kD fragments of the TSP chains associates with collagens at the same sites as intact TSP.

Discussion

TSP has been found in the extracellular matrix of a limited number of tissues and cultured cells (Jaffe et al., 1983, 1985; Mosher et al., 1982; Raugi et al., 1982; Wight et al., 1985), suggesting that it may be a relatively restricted or specific matrix component. As such TSP may impart some unique property to the matrices that contain it, and it is conceivable that, in combination with other matrix components, temporal or spatial refinements of the matrix might occur to suit specific functions during development or repair processes. To further examine the interaction of TSP with such matrix components, we undertook the study reported here, which extends with quantitative methods the initial report of Mumby et al. (1984). Using a semiquantitative ELISA assay (one to three plus) along with binding of labeled TSP to affinity matrices, these authors reported a preferential interaction of TSP with native type V collagen as compared with types I, III, and IV collagens. We have confirmed these results here in that in our quantitative direct binding assay we detected up to 10 times higher binding to type V collagen (Table I). Mumby et al. also noted that TSP bound to catalase and other proteins in their ELISA assay, which could not be detected with the affinity matrix assay. Since we are using TSP immobilized on plastic wells only for comparison of the various collagen types with one another, this unexplained effect should not be a problem in our study. Extending the

previous work we have shown that in a competition type assay, in which the different collagen types have equal access to TSP in solution, type III collagen also has reasonable affinity for TSP. Since we have used only pepsinized collagens in this study, these results reflect only interactions of TSP with the triple helical regions of these collagens.

The primary result of the direct binding studies is that while TSP can interact with all of the collagen types tested, there is a preferential reaction with type V collagen. This appears to be reflected mainly in the stability of the complex formed, such that it is more resistant to washing or dilution than the complexes of TSP with the other collagens. The effect of Ca on the association of TSP and the collagens is primarily one of shifting the binding curve to higher concentrations. It is impossible to determine whether this could represent a significant effect on matrix assembly in vivo, since all components may be brought together at a sufficiently high concentration that the Ca effect is not important. Alternatively, other ligands of TSP besides Ca may be able to exert an effect on the affinity of TSP for the collagens.

The specificity of the binding observed with the well plate assays is indicated by several factors. First, the binding of our iodinated TSP preparations to BSA-coated wells was always significantly less than the binding to any of the collagens. This nonspecific binding to BSA was routinely ∼1% of the total TSP in the assay, while binding to type V collagen was ∼25% of the input counts (see Table I). Second, heparin and fucoidin, both known ligands of TSP, inhibit the binding of TSP to type V collagen, whereas hyaluronic acid does not (Fig. 4). Third, mAb A6.1 is a good inhibitor of the binding of TSP to collagens, whereas other anti-TSP mAbs are up to 200-fold less potent. This inhibitory mAb recognizes the 70-kD collagen-binding domain of TSP on Western blots (Dixit et al., 1986*a*). Fourth, the collagen-binding domain of TSP, which lacks all of the globular regions of TSP, also binds to all types of collagen tested, and like intact TSP, binds preferentially to type V. Fifth, the microscopy of TSP–collagen complexes confirms the presence of specific sites on all of the collagen molecules, with the presence of an additional, positionally distinct specific site on type V collagen.

To examine the nature of the complexes formed between TSP and these collagens, we used a technique that has been used successfully by Furthmayr and his colleagues (Charonis et al., 1985) to demonstrate specific binding between laminin and type IV collagen, a case in which no specific binding could be detected with any direct binding assay. Sample preparation for rotary shadowing involves only the mixing of the two protein solutions in an appropriate buffer and then spraying the mixture onto mica chips. No dilution or washing is done, thus even low affinity or readily dissociable binding events can be observed. For the majority of this work we used the volatile ammonium bicarbonate buffer system; however, preliminary experiments gave similar results with dilute Tris buffers as well; thus it is unlikely that the ammonium bicarbonate buffer has induced artifactual binding events. The question of the specificity of the site at the end of the pepsinized collagen molecules was approached by examining complexes of the collagens and BSA, which is readily seen as a nearly spherical molecule in contrast to the rod-like collagens. These data (Fig. 7) shows an absolutely random distribution of BSA along the collagen molecules

with no suggestion of a preferred binding site at one end of the triple helix. Furthermore, these BSA "binding events" occurred with a much lower frequency than the TSP binding events.

Of particular interest is the finding that the trimer composed of the 70-kD fragments of TSP chains retains the ability to bind to both the end site on the collagens and the internal site on type V. It is not yet clear whether this 70-kD region contains two distinct binding sites, one for the end site on collagen and another for the internal site on type V, or if a single binding site on this region of TSP binds to both collagen sites. Recent cDNA cloning and sequencing studies (Dixit et al., 1986b; Frazier, W. A., S. W. Hennessy, and P. Rotwein, manuscript in preparation), have revealed a region near the amino terminus of the 70-kD domain that has significant homology with the cysteine-rich amino-terminal domain of the $\alpha 1(I)$ procollagen chain (Chu et al., 1984). This region corresponds precisely to exon 50 of the human αII gene (Chu et al., 1984). There are no stretches of Gly-X-Y structure in TSP, thus the homology disappears at the point at which this structure begins in the collagen chain. Nonetheless the structural data suggest that the TSP–collagen interaction may, in an evolutionary sense, be based on the prototype of the interaction of collagen chains with one another during their biosynthesis and assembly. The fact that the binding of the trimeric 70-kD domain to collagens shows no Ca dependence (Fig. 2) indicates that the collagen-binding structure of TSP is not inherently inhibited by Ca, but most likely resides near part of the structure that exhibits Ca-dependent conformational changes. The fact that mAb A6.1, which inhibits collagen binding, also binds to TSP in a Ca-dependent manner, further reinforces this idea. The amino acid sequence of TSP deduced from the cDNA (Frazier, W. A., S. W. Hennessy, and P. Rotwein, manuscript in preparation) contains a region after the collagen-binding domain composed of many repeating units containing a sequence that has several of the properties indicative of a calcium-binding structure such as occurs in the calmodulins and parvalbumins (Kretsinger, 1980).

The physiological relevance of the Ca-dependent conformational change in TSP is not yet known. The fact that TSP interacts with an extracellular molecule like type V collagen in a Ca-dependent way suggests the possibility that response of TSP to local changes in Ca concentration could affect the molecular structure or assembly of the extracellular matrix. In particular, Ca concentration could act to modulate matrix assembly via its effect on TSP conformation and subsequent changes in the interaction between TSP and type V or other collagens. Whether local Ca concentration can be regulated extracellularly as it can be intracellularly, is not known. However, it seems possible that sites within the extracellular matrix containing a high concentration of sulfated sugars could bind Ca ions and thus lower the free Ca concentration. On the other hand, extracellular Ca concentration need not play a role in regulation of assembly, since it is probable that the initial events of assembly, perhaps including the interactions of TSP with several collagen types, occur inside the cell in vesicles derived from the Golgi complex (Trelstad and Birk, 1984). It is feasible that regulation of Ca levels inside such vesicles could play a role in modulation of the assembly process before secretion of preformed bits of matrix material. While laminin and type IV collagen are recognized as major components of basement membrane, immunolocalization studies indicate the presence of TSP in basement membrane regions, particularly in blood vessels (Wight et al., 1985). The data reported here indicate that TSP can bind collagen types other than type V and raise the possibility that TSP may function to link type V collagen to other components of the basement membrane. The trimeric structure of TSP is ideally suited to such a role in both homotropic and heterotropic cross-linking of collagens. It remains to be determined if significant interactions exist between laminin and TSP.

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