Thrombospondin Modulates Focal Adhesions in Endothelial Cells

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Abstract. We examined the effects of thrombospondin (TSP) in the substrate adhesion of bovine aortic endothelial cells. The protein was tested both as a substrate for cell adhesion and as a modulator of the later stages of the cell adhesive process. TSP substrates supported the attachment of some BAE cells, but not cell spreading or the formation of focal adhesion plaques. In contrast, cells seeded on fibrinogen or fibronectin substrates were able to complete the adhesive process, as indicated by the formation of focal adhesion plaques. Incubation of cells in suspension with soluble TSP before or at the time of seeding onto fibronectin substrates resulted in an inhibition of focal adhesion formation. Furthermore, the addition of TSP to fully adherent cells in situ or prespread on fibronectin substrates caused a reduction in the number of cells, which were positive for focal adhesions, although there was no significant effect on cell spreading. In a dose-dependent manner, TSP reduced the number of cells with adhesion plaques to ~60% of control levels. The distribution of remaining adhesion plaques in TSP-treated cells was also altered: plaques were primarily limited to the periphery of cells and were not present in the central cell body, as in control cells treated with BSA. The observed effects were specific for TSP and were not observed with platelet factor 4, beta-thromboglobulin, or fibronectin. The TSP-mediated loss of adhesion plaques was neutralized by the addition of heparin, fucoidan, other heparin-binding proteins, and by a monoclonal antibody to the heparin binding domain of TSP, but not by antibodies to the core or carboxy-terminal regions of TSP. The interaction of the heparin-binding domain of TSP with cell-associated heparan sulfate appears to be an important mechanistic component for this activity of TSP. These data indicate that TSP may have a role in destabilizing cell adhesion through prevention of focal adhesion formation and by loss of preformed focal adhesions.

THE growth of anchorage-dependent cells is dependent on the ability of these cells to interact with a substrate which promotes physiological cell adhesion. Studies of in vitro model systems show that physiological cell substratum adhesion is a multistep process composed of several discrete stages (reviewed in references 3, 53). When a suspension of cells is seeded on a suitable substrate, the cells will initially attach to the substrate: if the substrate is composed of physiological adhesion molecules, attached cells will then proceed to flatten and spread with subsequent organization of specialized regions of the membrane and cytoskeleton into mature adhesion contacts, e.g., focal adhesion plaques. These plaques are involved in stabilizing cell substrate contacts. In recent studies, the molecular mechanisms involved in the adhesive process have begun to be elucidated. A family of receptors, the integrins, binds primarily to a RGD-containing sequence present in adhesive proteins. The interaction of integrins with substrate molecules appears to be required for cell attachment and spreading (reviewed in references 3, 16, 43, 45). Subsequent formation of focal adhesions requires an additional interaction between a cell associated heparan sulfate proteoglycan (50, 51) and a glycosaminoglycan binding site in the substrate molecule (26, 42, 52).

The process of physiological adhesion is dynamic and appears to be highly regulated. Focal adhesion plaques must disassemble during mitosis and when cells become motile and then re-form when cells return to the stationary state (3, 6, 53). Adhesion plaques are generally absent or reduced in highly migratory cells, whereas they are abundant in stationary anchorage-dependent cells (6). Similarly, transformed cells, which generally are more motile, have fewer and smaller focal adhesions than their nontransformed counterparts (3, 31). It is unclear how the assembly and disassembly of focal adhesion plaques are regulated. Growth factors, phorbol esters, proteases, and agents which increase cAMP levels have been implicated in focal adhesion disassembly (2, 3, 14, 15, 44).

Thrombospondin (TSP)[1] is a trimeric extracellular matrix glycoprotein which binds to cells through membrane-associated heparan sulfate proteoglycans (18, 35, 36, 41, 46). TSP contains a glycosaminoglycan-binding site and also a single RGDA sequence in each of three polypeptide chains (24). The RGD sequence in TSP has recently been shown to

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1. *Abbreviations used in this paper:* BAE, bovine aortic endothelial; TSP, thrombospondin; PF4, platelet factor 4.

bind to a species of integrins containing the beta-3 subunit (25). The ability of TSP to mediate cell attachment has been the subject of numerous studies (reviewed in references 12, 23) and there are several reports describing TSP-mediated attachment and spreading of transformed and undifferentiated cells (18, 39, 40, 48, 49). Data from several laboratories using different cell types suggest that both integrins and heparan sulfate proteoglycans can mediate initial cell attachment to TSP substrates (18, 25) and that sulfated glycolipids may be important for melanoma cell spreading on TSP (41). However, there are conflicting data as to whether TSP supports attachment and spreading of anchorage-dependent cells, such as endothelial and smooth muscle cells (5, 19, 20, 25, 34, 47). One report shows that these cells harvested with EDTA will attach to substrates coated with high concentrations of TSP (47), whereas, other investigators showed that trypsin-treated endothelial cells do not attach to substrates of TSP isolated from either human platelets or endothelial cell cultures (5). In addition, Murphy-Ullrich and Mosher (34) reported that TSP, either prebound to cells or preincubated with substrates, interfered with the ability of bovine aortic endothelial (BAE) cells to attach to plastic substrates coated with either type V collagen or fibronectin. This was also demonstrated by Lahav using TSP bound to fibronectin and glass substrates (19, 20).

In view of these apparently contradictory results, we decided to investigate the role of TSP in the later stages of substrate adhesion of BAE cells. In this study, we examined the ability of TSP to serve as a substrate for cell adhesion and the effect of TSP on the later stages of the cell adhesion process when the cells are attached to physiological substrates. Furthermore, we also studied the effects of TSP on these later stages of the adhesion process (spreading and extent and distribution of focal adhesions) in established cultures of BAE cells.

Materials and Methods

Materials

The following items were purchased: DME (Cell-Gro, Mediatech, Herndon, VA); FBS (HyClone Laboratories, Logan, UT); 500 μg/ml trypsin-2.2 mM EDTA (Flow Laboratories, McLean, VA); BSA, chondroitin sulfate (shark cartilage, mixed isomers), fucoidan, heparin (porcine intestinal mucosa), p-nitrophenol-N-acetyl-beta-D-glucosamide (N9376), cycloheximide, and glutaraldehyde, (Sigma Chemical Co., St. Louis, MO). Low molecular weight heparin (3,000 mol wt) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Monoclonal anti-vinculin antibody (clone VIN-II-5) was purchased from ICN Biochemicals (Lisle, IL), and NBD-phallicidin from Molecular Probes, Inc. (Eugene, OR). Rhodamine-labeled goat anti-mouse immunoglobulin antibody and normal mouse ascites control IgG were obtained from Cappel Laboratories (Malvern, PA). Monoclonal antibodies to specific domains of TSP were generous gifts of Dr. Vishva Dixit, University of Michigan (9-11, 13). In all procedures, heat denatured (80°C, 10 min, filtered) BSA was used.

TSP and Other Proteins

TSP, platelet factor 4 (PF4), beta-thromboglobulin, and human plasma fibronectin were generous gifts of Dr. Deane Mosher (University of Wisconsin, Madison). These proteins were purified according to previously published methods (32, 33, 35). Human fibrinogen from KABI-Vitrium AB (Stockholm, Sweden) was a gift of Dr. Lech Switalski, Department of Microbiology, University of Alabama at Birmingham. Contaminating fibronectin was removed from the fibrinogen by passing the protein solution through a column of gelatin-Sepharose. All proteins were pure as assessed by SDS-PAGE.

Cells

BAE cells (normal strain) were previously characterized (35). Cells were maintained in DME with 4.5 g glucose/liter, 2 mM glutamine, and supplemented with 20% FBS. Cultures were passed with trypsin-EDTA and used between passages 7 and 12. Testing for mycoplasma was routinely done and all cultures were negative.

Attachment Assays

Attachment assays were based on a method described by Lindgren (27) in which the number of cells attached to a substrate is determined by measuring the hexosaminidase activity of cells in a colorimetric reaction. Initial assays showed that the enzymatic response is linear in the range of 10^3–10^5 cells. Flat-bottom 96-well (model No. 3596; Costar Data Packaging Corp., Cambridge, MA) plates were coated with 100-μl solutions of the attachment proteins by incubating overnight at 4°C in a humidity chamber. Plates were rinsed twice with 0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl, 1 mM calcium chloride, 0.5 mM magnesium chloride (PBS+). Additional protein binding sites were blocked by incubating the wells for 30 min with 1% heat-denatured BSA in PBS+, followed by two rinses with PBS+. Cells to be used in the assay were harvested by treating the cell layers with trypsin-EDTA. When cells had detached, soybean trypsin inhibitor at a fivefold excess (wt/wt) was added, cells were then centrifuged (200 g), and resuspended in DME containing 0.1% BSA to a concentration of 0.4–6.0 × 10^5 cells/ml. Approximately 20 min elapsed between addition of soybean trypsin inhibitor and addition of 100 μl of cell suspensions to substrate-coated wells. Cells were then incubated with the substrates for 1 h at 37°C with 5% CO_2. Unbound cells were removed by washing three times with DME containing 0.1% BSA and once with PBS+. Plates were then incubated for 5 h at 37°C, with 60 μl/well of 5 mM p-nitrophenol-N-acetyl-beta-D-glucosamide in 0.5 M citrate buffer, pH 5.0, containing 0.25% Triton X-100. Color was developed by addition of 90 μl/well of 5 mM EDTA, 0.05 M glycine, pH 10.4, and read using a multiscan ELISA reader (Titertek, Elfab Dy, Finland) equipped with a 405-nM filter. Each sample was assayed in triplicate and experiments were performed at least twice.

Analysis of Cell Spreading and Focal Adhesion Formation

Cell spreading was assayed essentially as described by Woods et al. (52). Glass coverslips (12-mm-diam) were incubated with 100 μl of a protein solution containing fibronectin, fibrinogen, TSP, or heat-denatured BSA at a concentration of 100 μg/ml or PF4 and beta-thromboglobulin at 800 μg/ml and the slides were allowed to dry overnight or maintained in a humidity chamber at room temperature. Dried substrates were rehydrated by incubation with distilled water (100 μl for 30 min), followed by three 15-min washes with DME. Coverslips were placed in 24-well plates and additional protein-binding sites were blocked by incubating wells at room temperature for 30 min with 0.1% BSA in DME. Wells were washed three times with DME. Before harvesting with trypsin-EDTA, 75-mm² flasks of subconfluent BAE cells were treated for 2 h with 15 μg/ml cycloheximide. After harvesting, cells were treated with excess soybean trypsin inhibitor, centrifuged, and resuspended to ~1 × 10^5 cells/ml in DME with 15 μg/ml cycloheximide. This concentration of cycloheximide is sufficient to prevent 95% of protein synthesis by BAE cells (Dr. Richard LeBaron, Department of Biology, University of Alabama at Birmingham, unpublished results). 400–500 μl of cell suspensions were added to each coverslip per well and cells were allowed to attach and spread at 37°C, 5% CO_2 for 3 or 4 h. For viewing by interference reflection or phase microscopy, cells were fixed with 3% glutaraldehyde in DME (30 min, 37°C), rinsed three times with DME, and mounted on glass slides with PBS. In some experiments, medium was removed after the initial 3-4-h incubation, and cells were further incubated for up to 4 h with 400 μl/well of protein solution (TSP, BSA, PF4, or beta-thromboglobulin) in DME with 15 μg/ml cycloheximide. Alternately, cells and protein solutions were added together at the time of seeding on fibronectin-coated coverslips and incubated for 4 h at 37°C. In some experiments, cells were seeded on glass coverslips, grown overnight in DME with 20% FBS, treated for 2 h with 15 μg/ml cycloheximide, and then washed three times with DME before addition of proteins as described above. Cycloheximide-treated cells (10^6) were also incubated with TSP in suspension as previously described (35), washed three times with DME, and then seeded onto fibronectin-coated coverslips in the continued presence of cycloheximide and monitored for focal adhesions by interference reflection microscopy.

Spread cell area was determined after a 3-h incubation of cells on various

substrates or after a secondary incubation of cells with TSP subsequent to spreading. Cells were viewed by phase microscopy using a Nikon Optiphot microscope equipped with a drawing device and a digital planimeter system (Microplan II; Laboratory Computer Systems, Inc. Cambridge, MA).

Interference reflection microscopy using a Nikon Optiphot system was used to visualize focal adhesion plaques (17). To quantify the percent of cells having focal adhesions, a minimum of 100 spread cells per slide was examined. For a cell to be scored as positive, it had to have a minimum of three adhesion plaques present either in the central and/or peripheral regions of the cell. Spread cells were designated as cells having a nucleus recognizable by interference reflection microscopy or a noncircular shape.

Fluorescence Staining

Cells were treated as described above, except that cells were fixed with 3% formaldehyde in PBS for 10 min at 37°C, followed by three washes with DME. Cells were permeabilized by treatment with 0.1% Triton X-100 in PBS+ for 3 min at 4°C, washed three times with PBS+, followed by three 5-min rinses with 50 mM ammonium chloride, pH 7.3. Coverslips were washed twice with PBS+ before incubation with 0.1% BSA in PBS+ for 30 min to block sites of nonspecific binding. Coverslips were then incubated for 60 min with 100 μl of monoclonal anti-vinculin at a 1:10 dilution, followed by three washes with PBS+, and a 30-min incubation with rhodamine goat anti-mouse immunoglobulin used at ~50 μg/ml. After washing three times with PBS+, these cells were then stained for F actin using 1 U of NBD-phallicidin per coverslip for 20 min at room temperature. Coverslips were briefly washed three times with PBS+ before mounting on slides with PBS+. Double-labeled slides were viewed using a Nikon Optiphot microscope equipped with epifluorescence illumination. Normal mouse ascites IgG (10 μg/ml) was used as a negative control.

Results

TSP as a Substrate for Cell Attachment

TSP was tested for its ability to mediate attachment of BAE cells relative to substrates composed of fibronectin or fibrinogen, which are known to support BAE adhesion (7, 8). Attachment of cells to all of the substrates at 37°C was a time-dependent process, which was complete within 1 h (data not shown). Cell attachment to either TSP, fibronectin, or fibrinogen was proportional to the concentration of protein used to coat the wells (Fig. 1) up to coating concentrations of 3 μg/ml (0.3 μg/well): the number of cells attached did not increase when higher concentrations of these proteins were used to coat plates. The maximum number of cells attached to TSP substrates was 45% of the number of cells seeded, whereas a maximum of 90% of added cells attached to fibronectin or fibrinogen substrates. Less than 10% of seeded cells attached to BSA-coated wells (Fig. 1). Thus, while TSP supports attachment of BAE cells, it is not as effective as either fibronectin or fibrinogen substrates.

TSP Substrates Do Not Support BAE Spreading and Focal Adhesion Formation

The ability of BAE cells to spread on substrates of TSP relative to other substrates known to support BAE cell spreading was examined. BAE cells attached to coverslips coated with either 10 μg of fibronectin or fibrinogen underwent extensive spreading during a 3-h incubation period (Table I). These cells were characterized by the presence of focal adhesion plaques in 72-79% of the cells (Table I). The plaques were distributed throughout the cell body (Fig. 2, a and c). Staining for vinculin in cells spread on fibronectin similarly showed that vinculin was clustered into plaques which were present both over the central cell body and at the edges of
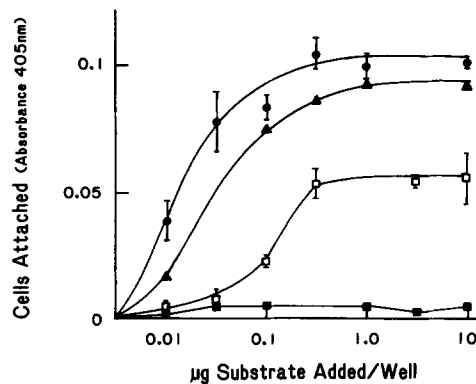
Figure 1. Attachment of endothelial cells to TSP substrates relative to fibronectin and fibrinogen substrates. Attachment assays were performed as described in Materials and Methods. Wells were coated with increasing concentrations of either fibronectin (●), fibrinogen (▲), TSP (□), or BSA (■). 2.2 × 10⁴ cells were added to each well and allowed to attach for 1 h at 37°C. The product of enzymatic activity was monitored at an absorbance at 405 nM and was proportional to the number of cells which remained bound after washings. Maximal attachment to fibronectin-coated wells was 90% of the cells added (2.0 × 10⁴ cells/well). In this assay, samples were assayed in duplicate on fibrinogen and BSA substrates and in triplicate on fibronectin and TSP substrates. Results are expressed as the means of replicate determinations. Standard deviations are given for samples performed in triplicate.

the cells (Fig. 3 a). Prominent stress fibers staining for F actin were present and traversed the cell body (Fig. 3 b). In contrast, cells did not spread well on TSP substrates: the area of cells attached to TSP was comparable to that of the few cells attached to BSA-coated coverslips (Table I) and there was almost no formation of focal adhesions or organization of vinculin into plaques (Figs. 2 d and 3 c). Staining for F actin in cells attached to TSP substrates showed that the actin-containing fibers were sparse, were primarily distributed circumferentially in cells, and did not appear to terminate end-on at the membrane (Fig. 3 d). Similarly, most cells were not fully spread on coverslips coated with other platelet glycoproteins, such as PF4 or beta-thromboglobulin, even when increased (80 μg) amounts of protein were used to coat coverslips (Table I). Interference reflection images of cells attached to either PF4 (Fig. 2 b), beta-thromboglobulin (data not shown), or the heparin-binding domain of fibronectin (52) resembled those of cells attached to TSP (Fig. 2 d). These cells developed close contacts that appeared as broad, gray areas by interference reflection microscopy (3, 21, 22).

To examine the possibility that drying of TSP or other heparin-binding proteins during the coating procedure altered the adhesive properties of the substrates, cell spreading experiments were repeated using coverslips coated with hydrated proteins. Cell spreading and focal adhesion formation was slightly enhanced on hydrated TSP or PF4 substrates as compared to adsorbed and dried substrates (Table I). However, these hydrated substrates were still much less effective in promoting spreading and focal adhesion formation than were fibronectin substrates.

TSP Inhibits the Formation of Focal Adhesions in Cells Seeded on Fibronectin Substrates

Incubation of cells in suspension with TSP at the time of

Table I. Effect of Substrate on Cell Spreading

Substrate	Cell area*	Cells spread‡	Cells with focal adhesions§
	μm^2	%	%
Adsorbed			
Fibrinogen	1,155 ± 599 (50)	99‖	72 (150)
Fibronectin	1,215 ± 552 (50)	96‖	79 (150)
PF4	467 ± 249 (50)	30	<1 (50)
Beta-thromboglobulin	363 ± 152 (20)	20	<1 (20)
Thrombospondin	328 ± 170 (101)	12	<1 (150)
Albumin	261 ± 119 (10)	10	<1 (10)
Hydrated			
Fibronectin	1,335 ± 659 (100)	94‖	63 (100)
Thrombospondin	475 ± 231 (100)	13	13 (100)
PF4	660 ± 321 (50)	16	17 (100)

* Cells were seeded in the presence of cycloheximide on coverslips adsorbed with the indicated substrates. After 3 h incubation, attached cells were fixed and spread cell area was measured using phase microscopy and a digital planimeter. Results are expressed as the mean cell area ± 1 SD. The number of cells counted are indicated in the parentheses.
‡ Cells with areas greater than the mean area of cells spread on fibronectin substrates minus one standard are considered to be spread. The minimal area of a cell spread on adsorbed substrates is 560 μm^2 and the minimal area on hydrated substrates is 690 μm^2.
§ Focal adhesions were detected by interference reflection microscopy. Numbers in parentheses indicate the number of cells counted.
‖ These values are significantly different from thrombospondin values ($P < 0.005$).
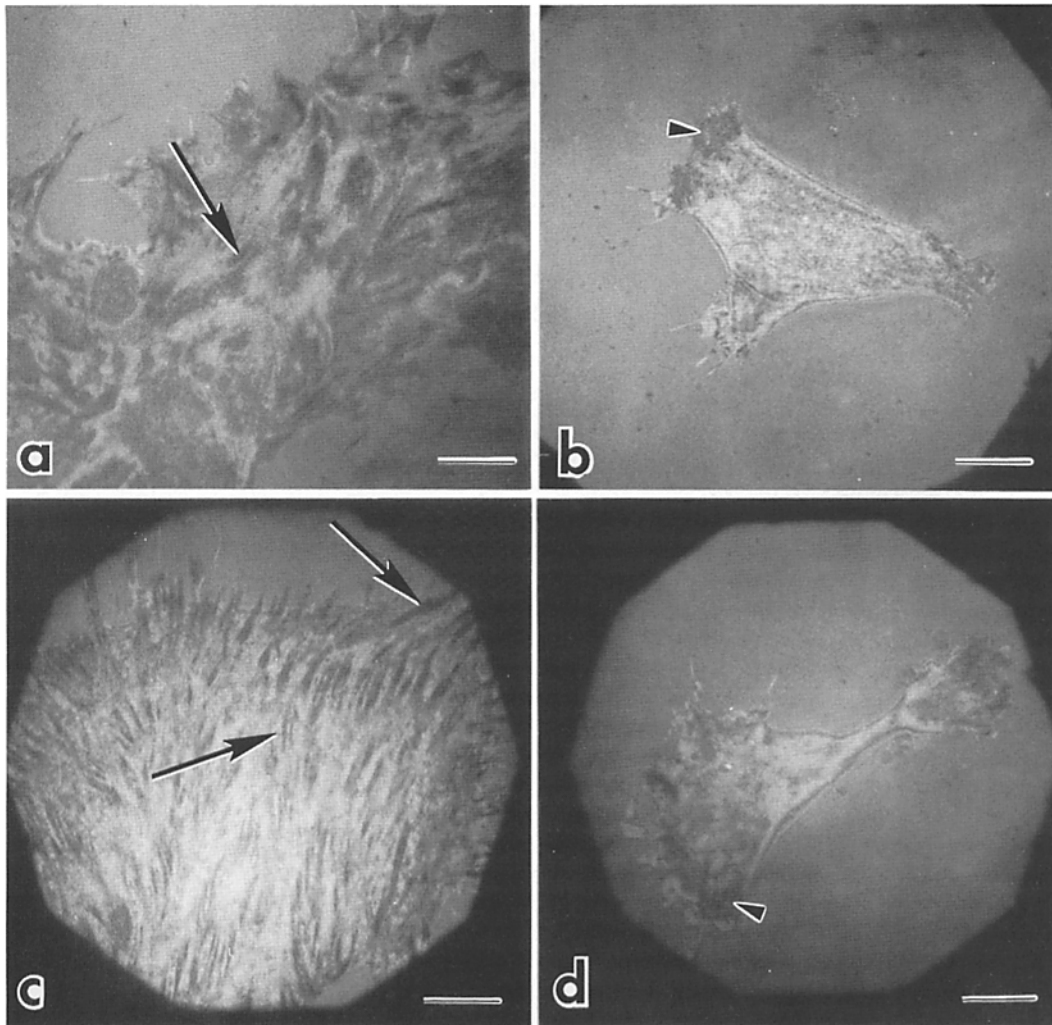
Figure 2. Cells attached to TSP do not form focal adhesion plaques. Cells attached and spread for 3 h in the presence of 15 µg/ml cycloheximide on coverslips adsorbed with 20 µg fibrinogen (*a*), 80 µg PF4 (*b*), 10 µg fibronectin (*c*), or 10 µg TSP (*d*). Cells were fixed and examined by interference reflection microscopy. Focal adhesions are present as dark streaks (*arrows*) in cells spread on either fibronectin or fibrinogen substrates, but are absent in cells spread on TSP or PF4. Cells spread on TSP and PF4 are slightly contracted and have broad lamellae with occasional gray areas of close contacts (*arrowheads*). Bars, 10 µm.
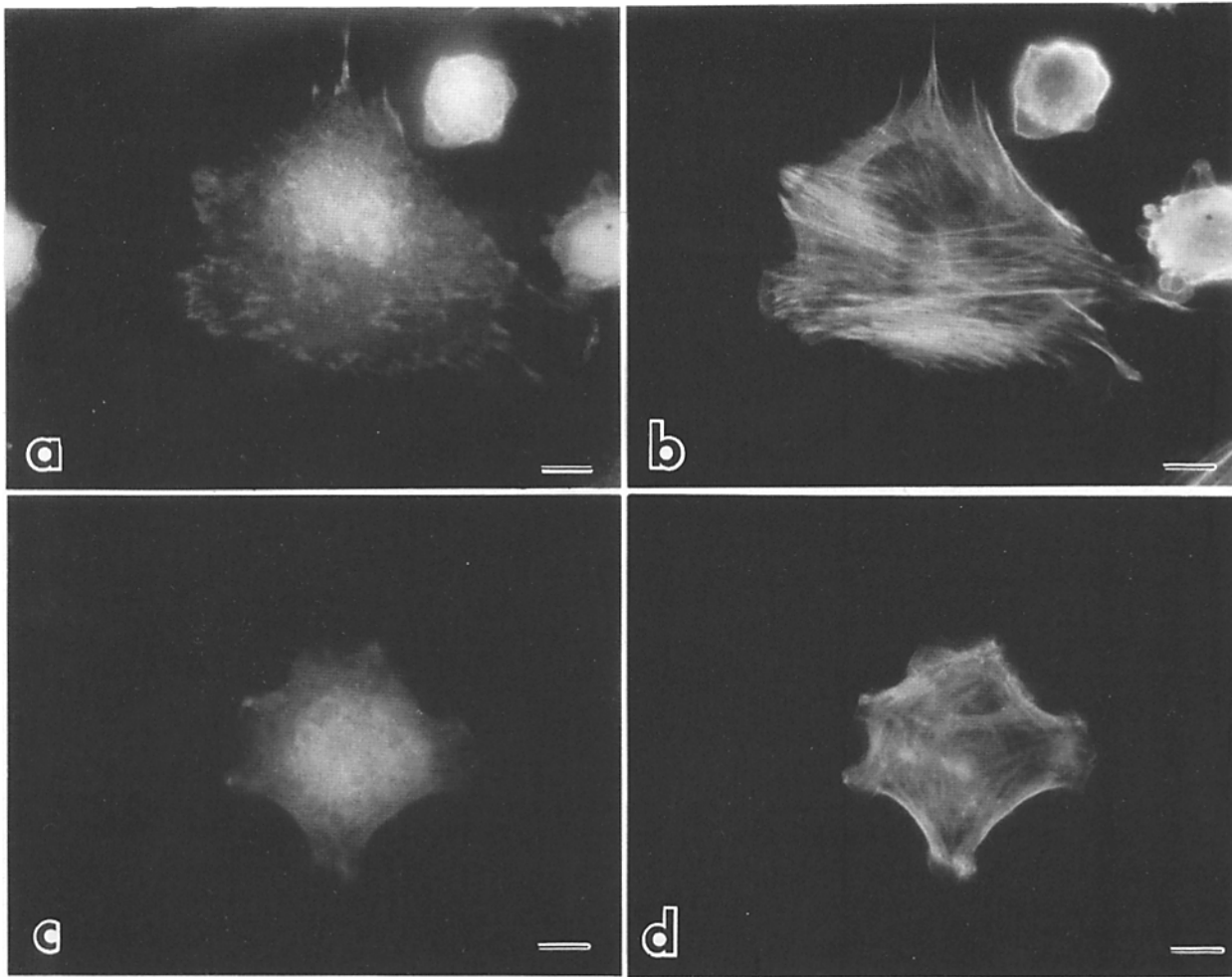
Figure 3. Localization of actin and vinculin in cells attached to fibronectin and TSP substrates. Cells were incubated for 4 h in the presence of 15 μg/ml cycloheximide on coverslips adsorbed with either 10 μg fibronectin (*a* and *b*) or 10 μg TSP (*c* and *d*). Coverslips were then fixed, permeabilized, and double-labeled by indirect immunofluorescence using a monoclonal antibody to vinculin and rhodamine-conjugated goat anti–mouse IgG (*a* and *c*) and by NBD-phallicidin binding to F actin (*b* and *d*). Cells spread on fibronectin contain plaque structures that stain for vinculin (*a*) and abundant stress fibers that stain for F actin (*b*). In contrast, cells attached to TSP generally show a diffuse staining of vinculin (*c*) and sparse actin-containing fibers are limited to circumferential regions of the cell (*d*). There is no positive staining in control slides in which normal mouse ascites IgG was substituted for the primary antibody (not shown). Bars, 10 μm.

seeding resulted in a slight reduction (~20%) of the number of cells attaching to a fibronectin substrate (34; data not shown). For the majority of cells that did attach, there were no apparent differences in the degree of spreading observed between cells pretreated with TSP and control cells treated with BSA (data not shown).

The number of cells forming focal adhesions on fibronectin substrates when seeded in the presence of TSP was greatly reduced as compared to control cells when examined by interference reflection microscopy (Table II). Cells were scored as positive or negative for focal adhesions according to the criteria described in Materials and Methods. The effect of TSP was dose-dependent with a maximum reduction of nearly 50% in the numbers of focal adhesion positive cells as compared to cells seeded in the presence of BSA. The half-maximal effect was observed at ~2–4 nM (1–2 μg/ml) TSP. Preincubation of cells with TSP before seeding also reduced the ability of cells to form focal adhesions. In these assays, cells were incubated in suspension with TSP for 1 h at 37 or 4°C, washed, resuspended, and allowed to attach and

spread on fibronectin substrates for 2 h (Table II). These results demonstrate that TSP affects the later stage of cell adhesion, e.g., focal adhesion formation during the process of adhesion to fibronectin substrates.

Treatment with TSP Causes a Loss of Focal Adhesions in Spread Cells

The possibility that TSP could affect not only the assembly of focal adhesions during the adhesion process but also the stability of preformed focal adhesions in fully spread cells was investigated. TSP was added to cells that had been allowed to attach, spread, and organize focal adhesions on fibronectin substrates during a 3–4-h period. TSP was incubated with these spread cells for 4 h and then examined by interference reflection microscopy for the presence of focal adhesion plaques. Results showed that there was a reduction in the number of TSP-treated cells having focal adhesion plaques which was dependent on the concentration of TSP added to the cells (Table III). Data collected from experi-

Table II. Effect of TSP on the Formation of Focal Adhesions

A. Focal adhesions in cells incubated with TSP during attachment and spreading

TSP	Percent of control
μg/well	
0.1	92
1	75
3	63
10	63
30	62
100	54

B. Focal adhesions in cells preincubated with TSP before seeding

Ligand	Percent of control
BSA	100
TSP (20 μg)	56
Heparin (1 μg)	100
Heparin and TSP	91

(*A*) 65% of control cells incubated with 30 μg BSA on fibronectin substrates formed focal adhesions. Results are expressed as percent of controls that contain focal adhesion plaques. A minimum of 100 cells were examined per condition. Coverslips were adsorbed with 10 μg FN. Increasing concentrations of TSP were added at the time cells, were seeded onto coverslips, and the cells were then incubated for 4 h at 37°C.
(*B*) Results are expressed as percent of control cells which were incubated with 30 μg BSA for 4 h at 37°C, washed, seeded on fibronectin-coated coverslips for 2 h at 37°C, and then examined for focal adhesion formation. 66% of control cells formed focal adhesions.

ments (*n* = 9) performed over several months showed that the percentage of cells with focal adhesions was reduced to 63% ± 9 SD of BSA-treated control cells. The maximal reduction observed was to 45% of control cells incubated with BSA. TSP at 10–100 μg/well was sufficient to cause a nearly maximal loss of focal adhesions in this system. Given that the number of controls positive for focal adhesions ranged from ∿65–79% of the total spread cells, this means that the actual percent of focal adhesion positive TSP-treated cells ranged from ∿32–45% of the total spread cells. The extent and dose response of TSP-mediated focal adhesion reductive activity were similar to the dose response obtained in assays testing the ability of TSP to prevent adhesion plaque formation.

In addition to reducing focal adhesions in cells spread on fibronectin, TSP also caused a dose dependent loss of focal adhesion plaques in cells in situ (Fig. 4). BAE cells were grown on glass coverslips in DME with 20% FBS until nearly confluent. Cycloheximide was added and cells were then exposed to increasing concentrations of TSP for 4 h (Fig. 4). The number of cells with focal adhesions was reduced in TSP-treated cultures (100 μg TSP) in a dose dependent manner to a maximum of nearly 50% of control cultures which were treated with cycloheximide and BSA. In eight separate experiments in which cells were exposed to 30 μg TSP, the average percent of focal adhesion positive cells relative to BSA-treated controls was 60 ± 5%.

Although TSP-treatment caused a reduction in the number of cells that were positive for focal adhesions, there were no significant differences in either the percent of cells spread or in the area of spread cells. In 10 separate experiments using

cells spread on fibronectin substrates, the percent of spread cells in TSP-treated samples was 93 ± 11% of the BSA-treated controls. Similar results were obtained from experiments using cells in situ. The spread cell areas of TSP-treated cells were similar (95% of control areas) to control cells. However, rare preparations of TSP did cause a reduction in cell spreading to 80% of controls, along with a more complete loss of focal adhesions to ∿22% of controls.

Interference reflection microscopy of TSP-treated cells spread on fibronectin substrates showed that in the cells that still had focal adhesions, the plaques that remained were generally fewer, shorter, and lighter gray in intensity (Fig. 5 *b*). The remaining plaques were primarily located at the cell edges with few, if any, focal adhesions located in the central portion of the cells (Fig. 5 *b*): this characteristic distribution was also observed in TSP-treated cells in situ. In contrast, control cells either seeded on fibronectin substrates or spread on native matrices in situ and incubated with BSA characteristically had adhesion plaques distributed in both central and peripheral regions of the cell (Fig. 5 *a*). The distribution and intensity of vinculin staining in TSP-treated, as well as, BSA control cells correlated with their respective interference reflection images (Fig. 6). In BSA-treated cells, vinculin plaques were abundant and were present both in the central cell body and at the cell edges (Fig. 6 *c*), whereas, in TSP-treated cells, vinculin staining plaques were sparse, thin, and primarily restricted to the cell edges (Fig. 6 *d*). Staining for F actin in TSP-treated cells demonstrated a corresponding tendency of actin microfilaments to be located primarily at the periphery of cells (Fig. 6 *f*), in contrast to the thicker stress-fibers which traversed the entire cell in BSA-treated controls (Fig. 6 *e*). Phase microscopy of spread cells (Fig. 6, *a* and *b*) incubated with TSP showed that these cells were more often somewhat spindle-shaped, had polarity, and frequently had broad cytoplasmic processes (Fig. 6 *b*).

The reduction in focal adhesions due to addition of TSP appeared to be complete within 60 min of incubation, al-

Table III. Effect of TSP on Loss of Focal Adhesions in Spread Cells

	Added protein	Percent of controls
	μg/well	
Thrombospondin	0.1	100
	1	81
	3	79
	10	65
	30	65
	100	62
PF4	1	99
	10	105
	30	97
Fibronectin	30	124
Beta-thromboglobulin	30	91

Results are expressed as percent of control cells with focal adhesions. Control cells were incubated with 30 μg BSA and the mean number of control cells with focal adhesions was 74%. A minimum of 100 cells were examined per condition. TSP or other proteins were added to cells seeded on substrates of 10 μg adsorbed fibronectin and allowed to spread for 3–4 h. Added proteins were incubated with cells for 4 h before fixation and microscopic examination.
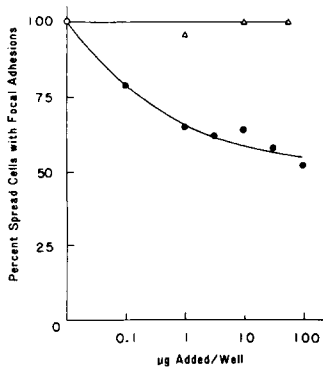
Figure 4. Treatment with TSP results in a dose-dependent reduction of focal adhesion plaques in cells in situ. Cells grown on coverslips were treated with increasing concentrations of TSP (●) or platelet factor 4 (△) for 4 h before fixation and examination by interference reflection microscopy. Control cells were treated with 30 μg BSA in 500 μl DME (○). A minimum of 100 spread cells per coverslip was examined to determine the presence of focal adhesion plaques. Results are expressed as the percent of cells with focal adhesions as compared to BSA treated control cells. 86% of BSA-treated controls had focal adhesion plaques. Cells incubated with either 30 μg fibronectin or 10 μg beta-thromboglobulin had 97% and 99%, respectively, of control levels of focal adhesions (not shown). All cells were pretreated with 15 μg/ml cycloheximide for 2 h before addition of proteins and further incubations were in the presence of cycloheximide.

though a slight further reduction was observed at 4 h of incubation with TSP (data not shown). It was previously shown that maximal binding of TSP to BAE cells occurred by 60 min of incubation at 37°C (35).

The reduction of focal adhesions was not due to a deleterious effect on cell viability resulting from prolonged cycloheximide treatment, since there was usually no more than a 5–10% decline in the number of cells scored as positive for the presence of adhesion plaques during the second incubation (1–4 h) with either PF4, BSA, or fibronectin, after the initial 3–4 h spreading on fibronectin substrates.

## The Loss of Focal Adhesions Is Induced Specifically by TSP

The observed reduction in focal adhesions in both cells in situ and in cells prespread on fibronectin was specific for TSP. Other platelet proteins, such as PF4 and beta-thromboglobulin, did not affect the number of cells with focal adhesions even when tested at molar concentrations nearly 15× higher than those effective for TSP (Fig. 4 and Table III). Addition of fibronectin in solution also did not reduce adhesion plaques, but instead resulted in slightly enhanced numbers of cells with adhesion plaques on fibronectin substrates (Table III). In addition, the number, distribution, and quality of focal adhesion plaques in individual cells were unaffected by the presence of PF4 (Fig. 5 c) or beta-thromboglobulin (not shown). The localization of vinculin plaques and the distribution of actin-containing stress fibers in cells treated with PF4 were indistinguishable from those of BSA-treated cells (data not shown).

The Heparin-binding Domain of TSP Is Essential for Activity

When heparin and TSP were co-incubated with BAE cells in suspension before seeding onto fibronectin, the effect of TSP on focal adhesion formation was abolished (Table II). Similarly, heparin neutralized the ability of TSP to reduce focal
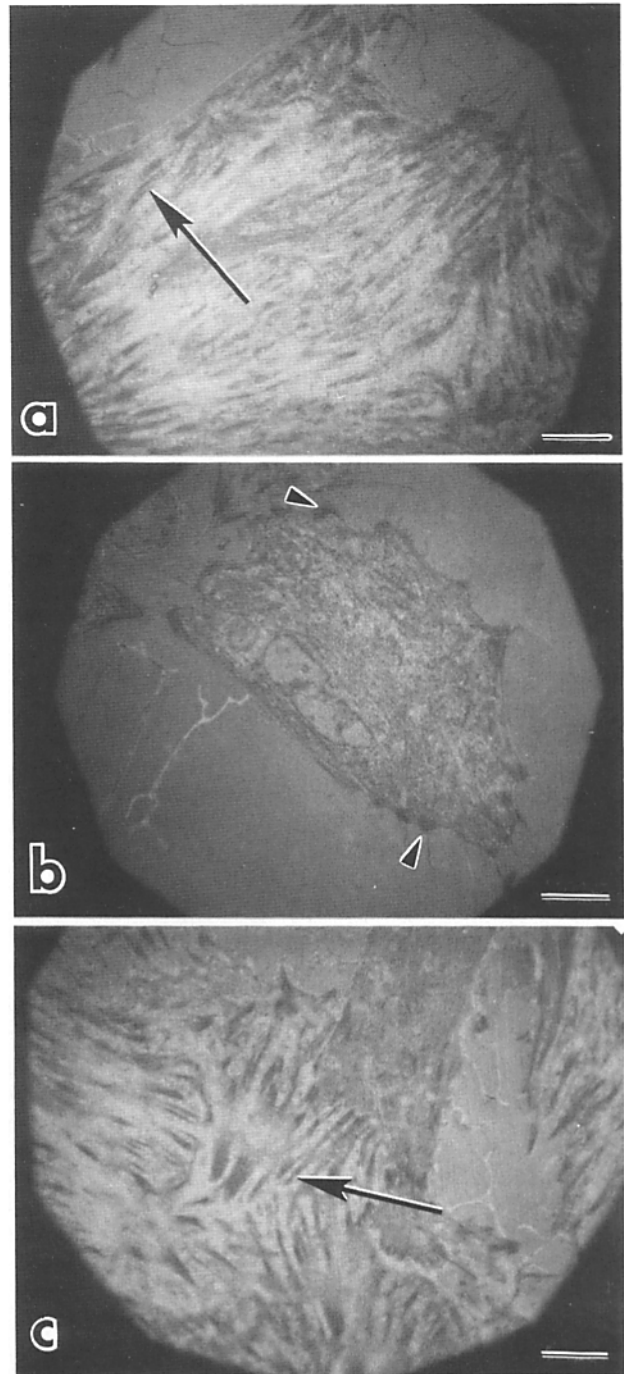

Figure 5. Interference reflection microscopy of spread cells incubated with soluble TSP. Cells spread for 4 h on fibronectin-adsorbed coverslips were then incubated for an additional 3 h with (a) 30 μg BSA, (b) 30 μg TSP, or (c) 30 μg PF4. Experiments were performed in the presence of 15 μg/ml cycloheximide. Interference reflection microscopy shows that spread cells incubated with BSA and PF4 have numerous focal adhesion plaques distributed throughout the cytoplasm (*arrows*); whereas, cells incubated with TSP which still have focal adhesions have fewer adhesion plaques which are predominantly located at the cell edges (*arrowheads*). Bars, 10 μm.
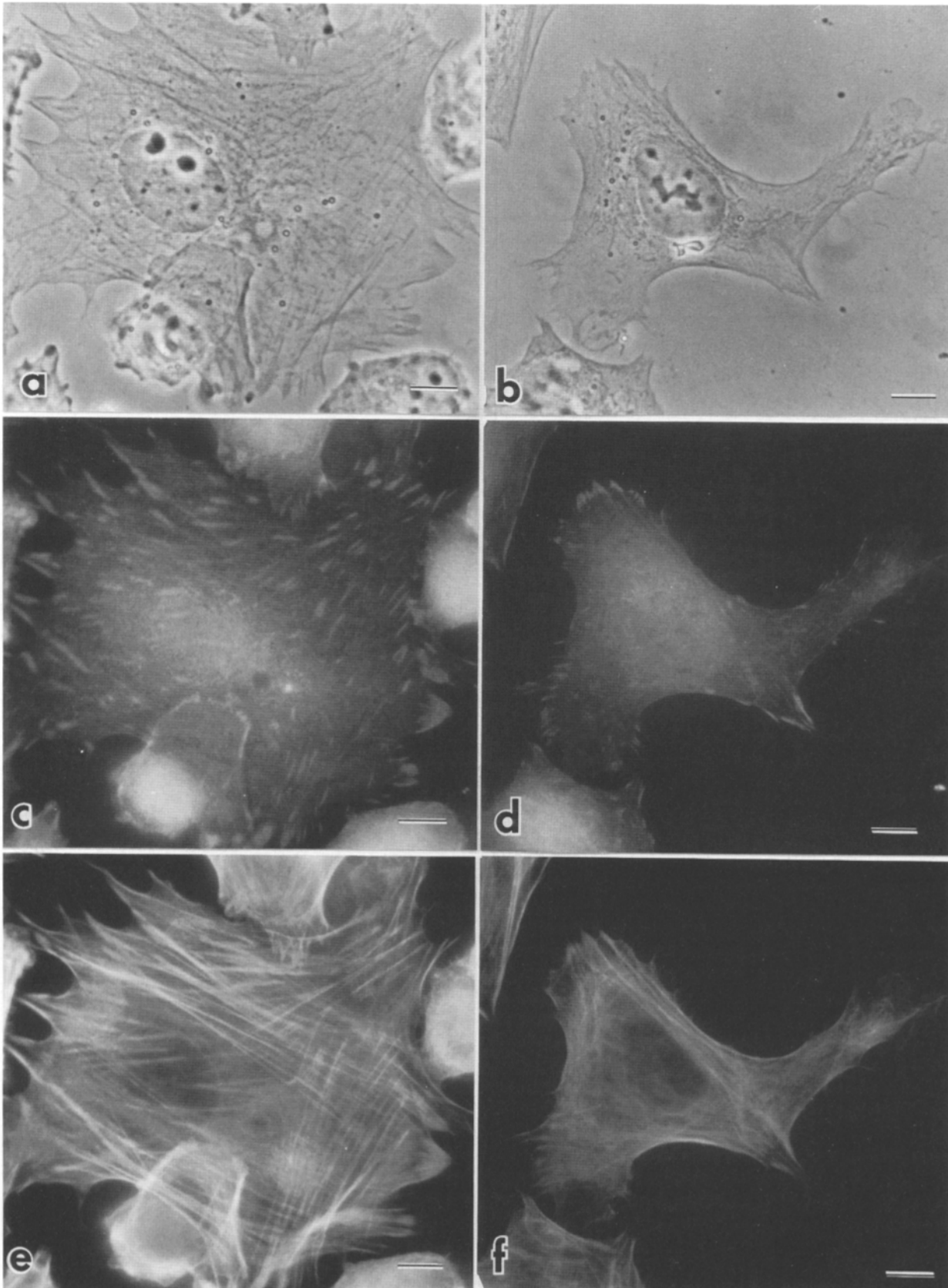
Figure 6. Distribution of vinculin and F actin in spread cells incubated with soluble TSP. Spread cells incubated with 30 μg BSA (*a, c,* and *e*) or 30 μg TSP (*b, d,* and *f*) were double-labeled for vinculin (*c* and *d*) or F actin (*e* and *f*) as in Fig. 3. Distribution of vinculin (*c*) and actin (*e*) in cells incubated with BSA is similar to that found in cells incubated with fibronectin for 4 h (Fig. 3). Cells incubated with TSP have small vinculin-containing plaques located at the edges of cells, but rarely in the central region of cells (*d*). The distribution of actin-containing fibers corresponds to the location of vinculin plaques: they are primarily located at the cell periphery (*f*). Phase microscopic images of these cells are shown in *a* and *b*. Bars, 10 μm.

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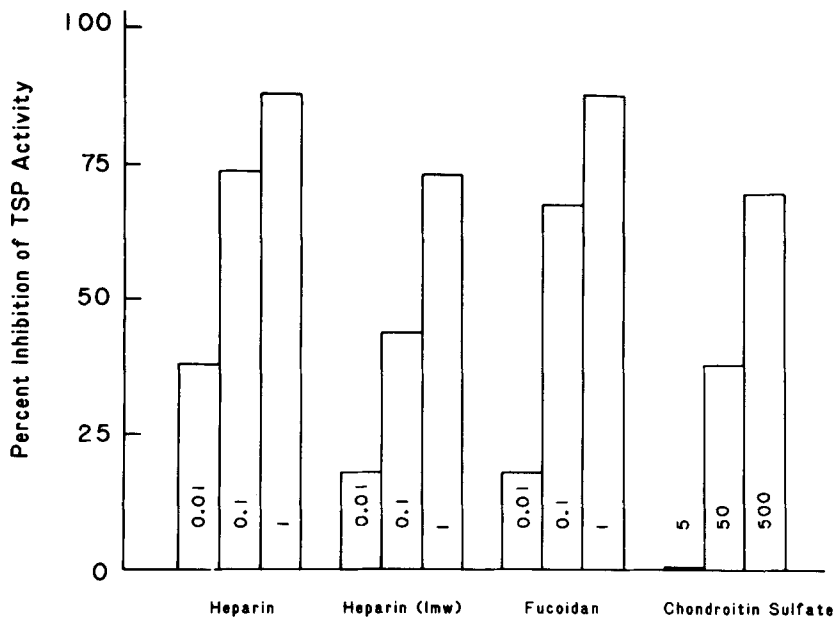
Figure 7. Inhibition of TSP-mediated loss of focal adhesions by heparin and fucoidan. Cells in situ were treated with 30 μg TSP in 500 μl DME and increasing concentrations of either heparin, low molecular weight heparin, fucoidan, or chondroitin sulfate. Concentrations of inhibitors are expressed in micrograms per milliliter. Cells were incubated with TSP and inhibitors for 4 h in the presence of cycloheximide, fixed, and examined by interference reflection microscopy. Cells treated with BSA (30 μg) were used as positive controls and the number of cells with focal adhesions in BSA-treated cells (68%) was set at 100%. Cells treated with 30 μg TSP alone had 50% of the number of cells with focal adhesions as compared to controls. Results are expressed as the percent inhibition in reduction of focal adhesions compared to cells incubated with TSP alone.

adhesions in cells grown on coverslips in situ (Fig. 7). Low molecular weight heparin also neutralized TSP activity, although somewhat higher concentrations of this form of the polysaccharide were required. In addition, other sulfated polysaccharides, such as fucoidan and chondroitin sulfate, blocked TSP activity. Fucoidan was effective at concentrations comparable to the heparins, whereas, at least 500-fold (wt/wt) more chondroitin sulfate, as compared to heparin, was required to inhibit TSP activity.

The sensitivity of TSP-mediated focal adhesion reduction to sulfated polysaccharides, such as heparin and fucoidan, suggests that the heparin-binding domain of TSP may be active in focal adhesion reduction. The localization of the active site was further analyzed by examining the ability of three domain-specific monoclonal antibodies to neutralize TSP activity. The active site appears to reside in the heparin-binding domain, since antibody A2.5, which is directed

against the amino-terminal heparin-binding domain of TSP, completely blocked the effect of TSP when tested at 10 μg/ml protein, whereas, monoclonal antibodies C6.7 and A6.1 directed against epitopes in the carboxy-terminal and core domains of TSP, respectively, did not prevent TSP-mediated focal adhesion loss (Table IV). Antibodies A2.5 and C6.7 tested alone had no significant effect on focal adhesion stability, however, antibody A6.1 did cause a slight reduction (30%) in focal adhesion positive cells.

TSP Interactions with Heparan Sulfate Appear to be Significant for TSP Activity

Previous studies have demonstrated that TSP binds to heparan sulfate located at the surface of Chinese hamster ovary, melanoma, mammary epithelial, and BAE cells (35, 36, 41, 46). It is therefore possible that the effect of TSP on focal adhesion formation and reduction is exerted via an interaction of the protein with heparan sulfate. Two other heparin-binding proteins, PF4 and beta-thromboglobulin, were tested for their ability to neutralize TSP-mediated loss of focal adhesions. These molecules compete for TSP binding to BAE cells (35). PF4 binds to heparan sulfate on endothelial cells and it has recently been shown that TSP and PF4 may interact with a common subspecies of heparan sulfate (4, 41). When tested in cells in situ, both proteins neutralized the ability of TSP to cause reduction of focal adhesion positive cells. PF4 (10 μg) blocked 100% and beta-thromboglobulin (10 μg) blocked 77% of TSP activity. These data, in concert with the fact that sulfated polysaccharides neutralize TSP activity, suggest that interactions of TSP with heparan sulfate are required to cause TSP-mediated reduction of focal adhesions.

Table IV. Inhibition of TSP-mediated Reduction of Focal Adhesions by Domain-specific Monoclonal Antibodies

	Percent control
TSP (30 μg)	45
BSA (30 μg)	100
TSP + mAb A2.5 (10 μg)	90
TSP + mAb A6.1 (10 μg)	44
TSP + mAb C6.7 (10 μg)	56
TSP + NS1 (ascites control)	56
mAb A2.5 (10 μg)	97
mAb A6.1 (10 μg)	70
mAb C6.7 (10 μg)	83
NS1	98

mAb A2.5 reacts with the NH₂-terminal heparin-binding domain of TSP; A6.1 reacts with the core-region; C6.7 reacts with the COOH-terminal platelet-binding domain. NS1 is normal mouse ascites. The antibodies were added together with TSP to prespread cells and incubated for 3 h. In BSA-treated controls, 71% of the cells formed focal adhesion plaques. Similar results were obtained using cells grown on coverslips and tested in situ.

Discussion

These studies show that TSP can serve as a substrate for endothelial cell attachment, but that TSP substrates are not competent to mediate cell spreading and focal adhesion for-

mation. In this respect, TSP acts similar to what has been observed for adhesion processes mediated by cell-associated heparan sulfate proteoglycans (21, 22). In fact, cell attachment to TSP substrates can be blocked by the addition of heparin, and cells deficient in heparan sulfate proteoglycans do not attach to TSP substrates (18). TSP-integrin interactions also support initial cell attachment (25), however, interactions of TSP with these receptors on endothelial cells do not appear to be sufficient to mediate the later stages of the complete cell adhesion process, cell spreading, and focal adhesion formation. This is consistent with the observations of Lawler et al. (25), who reported that endothelial cells attached to TSP substrates did not undergo spreading.

Not only do TSP substrates fail to promote physiologic adhesion, but soluble TSP either preincubated with cells before attachment or added to cells at the time of seeding prevents the formation of focal adhesion plaques. Furthermore, we show that soluble TSP added to adherent, fully spread cells, either in situ or on fibronectin substrates, causes a reduction in the number of cells with focal adhesion plaques without significantly affecting cell spreading.

The effect of TSP was not limited to BAE cells, since we observed a similar reduction in the number of cells with focal adhesions in experiments using human embryo fibroblasts (data not shown).

It is not known what molecular mechanisms are involved in TSP-mediated inhibition of focal adhesion formation and in focal adhesion disassembly. We cannot exclude the possibility that the loss of focal adhesions in prespread cells is due to TSP inhibition of re-formation of focal adhesions, rather than to the effect of TSP inducing disassembly of formed focal adhesions. In these experiments, the ability of TSP to either prevent focal adhesion formation or to cause a reduction in focal adhesions was only partial. This may reflect heterogeneity within a population of cells with respect to TSP-binding and degradation (see Fig. 6 in reference 36), as well as, heterogeneity in focal adhesion stability and turnover.

In this study, the active site has been located to the heparin-binding domain of TSP and binding to cell-associated heparan sulfate seems to be an important step in the inhibitory and/or reductive process, since the presence of sulfated polysaccharides and other heparin-binding proteins, PF4 and beta-thromboglobulin, interfere with the action of TSP. It is reasonable that PF4 neutralizes TSP activity by competing for cell-associated heparan sulfate, since it has recently been reported that TSP and PF4 bind to overlapping populations of heparan sulfate molecules from melanoma cells (41). The formation of focal adhesions by fibroblasts and Chinese hamster ovary cells has been shown to be dependent upon a dual receptor–ligand interaction, involving a receptor of the integrin type and a cell-associated heparan sulfate proteoglycan (26, 52). It is therefore not surprising that blocking of the heparan sulfate proteoglycan with a soluble ligand (e.g., TSP) may interfere with the process of focal adhesion formation and reduction. However, binding of a ligand to cell-associated heparan sulfate does not automatically lead to loss of focal adhesions, since addition of PF4 and beta-thromboglobulin to fully spread adherent cells did not result in a reduction of focal adhesion plaques. Therefore, there appear to be properties of TSP, distinct from its heparin-binding activity, that are active in preventing focal adhesion formation

and causing loss of focal adhesions. It is possible that a second site on the TSP molecule is mechanistically important.

The assays in which addition of TSP caused focal adhesion loss in cells in situ show that the TSP effect on focal adhesions is not simply due to selective trypsin sensitivity of various matrix receptors which may potentially be cleaved during cell harvesting before seeding onto fibronectin substrates. Furthermore, these data show that TSP is active on cells spread on their native matrices.

TSP appears to preferentially affect adhesion plaques located in the central regions of the cells: plaques at the cell periphery are refractory to the presence of TSP, at least during the course of these experiments. The significance of this observation is not clear, however, there may be heterogeneity in the temporal stability, structure, or regulation of adhesion plaque formation/disassembly between focal adhesions located in various parts of the cell. There is some evidence for such heterogeneity: Dejana et al. (8) recently showed that endothelial cells spread on vitronectin initially (within 30 min) formed plaques only at the cell edges, and later, adhesion plaques also appeared in the central regions of the cell. It has also been shown that transformed cells have "dot"-type focal adhesions at the cell edges, whereas their nontransformed counterparts have focal adhesions in both the central and peripheral regions of cells (1, 31).

The data presented in this report suggest that TSP destabilizes cell matrix contacts. This function may correspond to the apparent temporal availability of TSP in tissues. TSP is released in large amounts from platelets after injury and platelet aggregation. Transcription of TSP mRNA and synthesis of TSP are increased rapidly, although transiently, in the presence of growth factors (29, 30, 37). It is also a transient component of clot matrices (33) and is present during the early stages of wound healing (38). Similarly, the binding of TSP is increased in subconfluent as compared to density-arrested cells (34). The interaction of TSP with cells is short lived, as it is rapidly degraded by the cells (28, 34–36). Thus, based on these data, it is reasonable to suggest that TSP functions in processes such as wound healing, angiogenesis, and development by "priming" cells for mitosis or migration by destabilizing cell matrix contacts through the loss of focal adhesion plaques.

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