Cell-Binding Domain of Endothelial Cell Thrombospondin: Localization to the 70-kDa Core Fragment and Determination of Binding Characteristics†

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ABSTRACT: Endothelial and other cell types synthesize thrombospondin (TSP), secrete it into their culture medium, and incorporate it into their extracellular matrix. TSP is a large multifunctional protein capable of specific interactions with other matrix components, as well as with cell surfaces, and can modulate cell adhesion to the extracellular matrix. With the aim of understanding the mechanism by which TSP exerts its effect on cell adhesion, we studied the interaction of endothelial cell TSP (EC-TSP) with three different cell types: endothelial cells, granulosa cells, and myoblasts. We find that endothelial cells specifically bind radiolabeled EC-TSP with a K_d of 25 nM, and the number of binding sites is 2.6×10^6/cell. Binding is not inhibitable by the cell-adhesion peptide GRGDS, indicating that the cell-binding site of EC-TSP is not in the RGD-containing domain. Localization of the cell-binding site was achieved by testing two chymotryptic fragments representing different regions of the TSP molecule, the 70-kDa core fragment and the 27-kDa N-terminal fragment, for their ability to bind to the cells. Cell-binding capacity was demonstrated by the 70-kDa fragment but not by the 27-kDa fragment. Binding of both intact [^{125}I]EC-TSP and of the ^{125}I-labeled 70-kDa fragment was inhibited by unlabeled TSP, heparin, fibronectin (FN), monoclonal anti-TSP antibody directed against the 70-kDa fragment (B_{7-3}), and by full serum, but not by heparin-absorbed serum or the cell-adhesion peptide GRGDS. The 70-kDa fragment binds to endothelial cells with a K_d of 47 nM, and the number of binding sites is 5.0×10^6/cell. Rat granulosa cells, which synthesize and secrete TSP, can also bind both intact EC-TSP and the 70-kDa fragment with binding features very similar for the two ligands. That is, the binding of both ligands is inhibited to a similar extent by unlabeled EC-TSP, by the monoclonal antibody B_{7-3}, and by FN, but not by the GRGDS peptide. In the case of granulosa cells, heparin did not inhibit binding of EC-TSP or the 70-kDa fragment. Granulosa cells bind intact EC-TSP and the 70-kDa fragment with dissociation constants of 1.8 and 38 nM, respectively, indicating a significant reduction in affinity for TSP upon fragmentation. The number of binding sites on granulosa cells is 1.7×10^5/cell for the intact protein and 2.5×10^5/cell for the 70-kDa fragment. In contrast to the TSP-synthesizing endothelial and granulosa cells, primary rat myoblasts, which do not synthesize TSP, fail to bind EC-TSP. Our observations suggest that (a) TSP synthesis correlates with the ability of a given cell type to bind TSP and (b) EC-TSP binding to the TSP-synthesizing cell is mediated by a domain contained in the 70-kDa core fragment. The mechanism of binding, however, may vary from one cell type to another, as suggested by the differential effect of heparin on TSP binding by endothelial and granulosa cells.

Thrombospondin (TSP), a 450-kDa glycoprotein, is synthesized, secreted, and incorporated into the extracellular matrix of endothelial cells and various other cells in culture (McPherson et al., 1981; Raugi et al., 1982; Mosher et al., 1982; Jaffe et al., 1983, 1985; Asch et al., 1986; Dreyfus & Lahav, 1988; Canfield et al., 1989; Kreis et al., 1989; Clezardin et al., 1989). In vivo, it has been detected in several human tissues (Wight et al., 1985; Pratt et al., 1989). TSP is also present in the α-granules of resting platelets and is secreted following stimulation (Lawler et al., 1978).

TSP interacts with a variety of macromolecules, among them heparin (Lawler & Slater, 1981), fibronectin (Lahav et al., 1982, 1984; Dardik & Lahav, 1989), collagen (Lahav et al., 1982; Mumby et al., 1984), and sulfated glycolipids (Roberts et al., 1985). Once deposited into the extracellular matrix, TSP codistributes with other matrix components, for which it was shown to have an affinity (Dreyfus & Lahav, 1988). TSP synthesis and matrix incorporation is regulated by cell density (Mumby et al., 1984), age (Kramer et al., 1985), and induction of heat shock (Ketis et al., 1988) and can be specifically augmented by platelet-derived growth factor (Majack et al., 1985). TSP has been shown to support surface attachment of several tumor cell lines (Varani et al., 1986; Roberts et al., 1987; Tuszynski et al., 1987) but not that of normal cells (Lahav, 1988a,b; McClenick, 1989) or of other cancer cell lines (Clezardin et al., 1989). Binding of TSP to smooth muscle cell surface is required for cell proliferation (Majack et al., 1988). These findings suggest heavy involvement of TSP in matrix construction, as well as in regulation of cell adhesion, growth, and development.

Several groups have analyzed the interaction of platelet-derived TSP with various cell types. TSP was shown to interact with both resting and stimulated platelets (Wolff et al., 1986), monocytes and macrophages (Silverstein & Nachman, 1987), fibroblasts (McKeown-Longo et al., 1984), endothelial cells (Murphy-Ullrich & Mosher, 1987), keratinocytes (Varani et al., 1988), and several tumor cell lines (Clezardin et al., 1989; Varani et al., 1986; Roberts et al., 1987; Riser et al.,

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1988). Different monoclonal anti-TSP antibodies were used in an attempt to localize the cell-binding domain of platelet TSP. Melanoma cell attachment to TSP was shown to be inhibited by three monoclonal antibodies directed against three different epitopes on TSP, suggesting that TSP can interact with cell surfaces at multiple sites along the molecule (Roberts et al., 1987; Riser et al., 1988). The interaction of cells with FN was also shown to occur at multiple sites along the FN molecule (Akiyama et al., 1985).

The cell-adhesion sequence RGD, which mediates cell adhesion of various adhesive proteins such as a fibronectin and fibrinogen (Ruoslahti & Pierschbacher, 1986; Hynes, 1987), is also present in TSP (Lawler & Hynes, 1986). A recent report shows that endothelial cells, smooth muscle cells, normal rat kidney cells, and the monocyte-like U937 cells utilize the RGD sequence for attachment to TSP (Lawler & Hynes, 1988). However, numerous studies have demonstrated RGD-independent attachment of various cell types, e.g., melanoma (Roberts et al., 1987) and squamous carcinoma cells (Varani et al., 1988), to TSP. The existence of both RGD-dependent and RGD-independent mechanisms of cell–TSP interaction further corroborates the ability of TSP to interact with cells at multiple sites and suggests, in addition, that different cell types differ in the mechanism of TSP recognition.

As mentioned above, most of the studies on TSP–cell interaction were performed with platelet TSP. We, as well as others, have found significant structural differences between platelet TSP and endothelial cell TSP (EC-TSP), which may point to the existence of functional differences between the molecules of the two sources (Dardik & Lahav, 1987, 1989; Clezardin et al., 1986). Indeed, we found that the two molecules differ in the number of binding domains to both heparin (Dardik & Lahav, 1987) and fibronectin (Dardik & Lahav, 1989).

In an attempt to understand the mechanism by which cellular TSP interacts with cell surfaces and exerts its effect on cell adhesion, we studied the interaction between EC-TSP and three different cell types: endothelial cells, granulosa cells, and myoblasts.

Our data, presented here, demonstrate that TSP binding capacity depends on the ability of a particular cell type to synthesize TSP, that different mechanisms exist for TSP binding by different cell types, and finally, using limited proteolytic fragmentation and a monoclonal anti-TSP antibody (B_{7-3}), that the cell-binding domain of EC-TSP is localized to the 70-kDa trypsin-resistant core fragment. Aspects of this study have been presented previously (Lahav & Dardik, 1990).

Materials and Methods

Cells. Bovine aortic endothelial cells were a kind gift from M. A. Gimbrone. They were grown and maintained as previously described (Gimbrone et al., 1974). Rat granulosa cells and rat primary myoblasts were kindly provided by Dr. Abraham Amsterdam from the Department of Hormone Research and by Dr. Zvi Vogel from the Department of Neurobiology, respectively, at The Weizmann Institute of Science, Rehovot.

Metabolic Labeling of Bovine Aortic Endothelial Cells. Radiolabeling of secreted material was done by metabolic incorporation of radioactive methionine under the conditions described earlier (Dardik & Lahav, 1987). The cells were grown to near confluence in 10-cm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum depleted of TSP and fibronectin by passage over heparin-Sepharose. Cultures were then labeled by incubation for 24 h in medium containing 25 μCi/mL [^{35}S]methionine (Amersham) in DMEM containing one-tenth the regular concentration of methionine and 10% bovine serum passed over heparin-Sepharose. The culture medium was then collected and centrifuged for 15 min at 5000g at 4 °C; phenylmethanesulfonyl fluoride (PMSF) was added (2 mM final concentration), and the medium was kept frozen at −80 °C until further use.

Isolation of Metabolically Labeled Endothelial Cell Thrombospondin (EC-TSP). EC-TSP was purified as described earlier (Dardik & Lahav, 1987). Briefly, culture medium containing metabolically labeled secreted proteins (Dardik & Lahav, 1987) was first depleted of fibronectin by passage over gelatin-Sepharose. The effluant was collected and passed over a heparin-Sepharose column preequilibrated with Ca^{2+}-containing Tris/NaCl (0.15 M NaCl, 10 mM, NaN_3, 20 mM Tris/HCl, pH 7.6). The heparin column was then washed with Tris/NaCl followed by 0.25 M NaCl in the same buffer. Finally, [^{35}S]TSP was eluted with 0.55 M NaCl in the same buffer, frozen, and stored at −80 °C.

Proteolytic Fragmentation and Purification of EC-TSP Fragments. Metabolically labeled EC-TSP was dialyzed against Tris/NaCl prior to proteolysis. TSP depleted of Ca^{2+} was prepared by adding EDTA, at a final concentration of 5 mM, to samples of TSP containing Ca^{2+}.

Limited proteolysis of TSP and identification of the fragments produced were carried out as described earlier (Dardik & Lahav, 1987, 1989) Metabolically labeled EC-TSP (2–5 mL of 01.–0.5 mg/mL protein) was proteolyzed with chymotrypsin in the presence of EDTA for 5 min at 22 °C, and the digest containing fragments of 27- and 70-kDa fragments (Dardik & Lahav, 1989) was passed over 1 mL of heparin Sepharose. The 27-kDa fragment (i.e., the amino-terminal fragment) bound quantitatively to the column, whereas most of the 70-kDa fragment (the proteolysis resistant core fragment) came out in the effluent (Dardik & Lahav, 1989). The effluent was, therefore, used in cell-binding experiments without further purification of the 70-kDa fragment. The bound 27-kDa fragment was further purified by removal of the bound 70-kDa fragment with 0.25 M NaCl prior to elution with 0.55 M NaCl (Dardik & Lahav, 1989).

Radioimmunoprecipitation. A 400-μL Staph-A suspension (Pansorbin, Calbiochem) was centrifuged for 5 min at 10000g, washed three times with immunoprecipitation buffer (PBS containing 0.02% NaN_3, 1% Triton X-100, 0.1% SDS, and 0.1% BSA, pH 8.6), and incubated with 400 μL of goat anti-mouse IgG + IgM (Sigma) for 30 min at 22 °C. Following incubation, the suspension was centrifuged, washed three times in the above buffer, resuspended to a final volume of 400 μL in the same buffer, and divided into two aliquots of 200 μL. To produce precipitins, one of the aliquots was incubated with 1 mL of hybridoma culture medium (DMEM supplemented with 10% horse serum) and the other with 1 mL of hybridoma culture medium containing the monoclonal anti-TSP antibody B_{7-3} for 40 min at 22 °C. Following incubation, the precipitins were centrifuged and washed three times with immunoprecipitation buffer, and each was divided into two samples. Each sample of precipitin was then centrifuged and the pellet resuspended in 100 μL of an antigen solution containing either intact [^{35}S]EC-TSP in Tris/NaCl including 5 mM EDTA or the chymotryptic digest of [^{35}S]-EC-TSP produced in the presence of EDTA as described above. The incubation with the antigen solution was carried out for 16 h at 4 °C. The samples were then washed four times with immunoprecipitation buffer and bound material was released by boiling the pellet for 3 min in electrophoresis

sample buffer. The material thus eluted was analyzed by SDS–PAGE.

Iodination of Proteins. Proteins were radiolabeled by using two Iodobeads (Bio-Rad Laboratories, Richmond, CA) with 0.5 mCi $Na^{125}I$ (Amersham) per mL of protein solution (50–100 μg/mL) according to the manufacturer's instructions. The reaction was stopped by removal of the Iodobeads, and the iodinated protein was separated from free iodine by gel filtration on a Sephadex G-25 column (Pharmacia). A specific activity of (1–5) × 10^5cpm/μg of protein was obtained with this procedure.

Binding Assays. Binding assays were performed in suspension. Bovine aortic endothelial cells, rat granulosa cells, or rat myoblasts were harvested with PBS containing 5 mM EDTA, washed with DMEM, resuspended in DMEM containing 0.2% BSA, and divided into 200-μL aliquots of (0.2–1) × 10^5 cells/sample. In those experiments in which the effect of serum was studied, the cells were resuspended in DMEM supplemented with either 10% full bovine serum or 10% of the same serum passed over heparin-Sepharose. In dose-dependence experiments, the cells were incubated with varying amounts of [^{125}I]EC-TSP, ^{125}I-labeled 70-kDa fragment or ^{35}S-labeled 27-kDa fragment for 60 min at 22 °C. In inhibition experiments, each sample was incubated with 1 μg/mL [^{125}I]EC-TSP or ^{125}I-labeled 70-kDa fragment, and various inhibitors were added as described in Table 2. Following incubation, the cells were washed twice with PBS by centrifugation for 1 min at 10000*g* and resuspended in 0.2 mL of PBS, and the cell-bound radioactivity was counted in a γ-counter (iodinated samples) or in a β-scintillation counter (^{35}S-labeled samples). Nonspecific binding of iodinated ligands was determined according to Murphy-Ullrich and Mosher (1987) by adding the [^{125}I]EC-TSP in the presence of 10 μg/mL heparin, in the case of endothelial cells, and in the presence of 50 μg/mL unlabeled EC-TSP in the case of granulosa cells. Unless stated otherwise, data shown in the figures represent specific binding, i.e., the difference between the total binding and the nonspecific binding. The latter varied between 20 and 40% of the total binding (Murphy-Ullrich & Mosher, 1987).

SDS–Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out on slab gels using the discontinuous system according to Laemmli (1970). Unless otherwise stated, samples to be analyzed were boiled in electrophoresis sample buffer containing 2% SDS, 2 mM EDTA, 10% glycerol, 0.1 M dithiothreitol, and 80 mM Tris, pH 6.8. Molecular weight calibration was done with reduced fibronectin (220000), myosin (200000), and the low molecular weight markers kit (Pharmacia Fine Chemicals; the molecular weights of the polypeptides in the kit are 94000, 68000, 43000, 30000, 20100, and 14400). After electrophoresis, protein bands were detected by staining the gels with 1% Coomassie brilliant blue R (Sigma) in 50% methanol and 10% acetic acid. For detection of radiolabeled proteins, gels were impregnated with Autofluor (National Diagnostics), dried, and incubated with Agfa Gevart X-ray film at −80 °C according to Bonner and Laskey (1974).

Determination of Binding Constants. Dissociation constants (K_d) were determined by graphical analysis in double-reciprocal plots of 1/[bound ligand] vs 1/[free ligand], which yielded straight lines (Bing et al., 1982; Dardik & Lahav, 1989).

Results

Binding of EC-TSP to Bovine Aortic Endothelial Cells. Endothelial cells bind [^{125}I]EC-TSP in a dose-dependent and saturable manner (Figure 1). Maximal inhibition (~70%) was obtained by addition of 10 μg/mL heparin to the incubation medium (Figure 1). No increase in the extent of inhibition could be achieved by increasing heparin concentration up to 100 μg/mL (Figure 1). It was therefore possible to use heparin at the concentration of 10 μg/mL to determine the level of nonspecific binding as described by Murphy-Ullrich and Mosher (1987). The same level of nonspecific binding was observed when the incubation was carried out at 22 or 4 °C (Figure 1), excluding TSP internalization as a possible reason for the observed nonspecific binding. Double-reciprocal analysis of the data showed that the plot of 1/[bound ligand] vs 1/[free ligand] gave a straight line (data not shown). This enables calculation of the dissociation constant and the number of binding sites for EC-TSP on endothelial cells as described (Lahav et al., 1984; Dardik & Lahav, 1987, 1989; Bing et al., 1982), provided there is no degradation of EC-TSP following binding (see below).

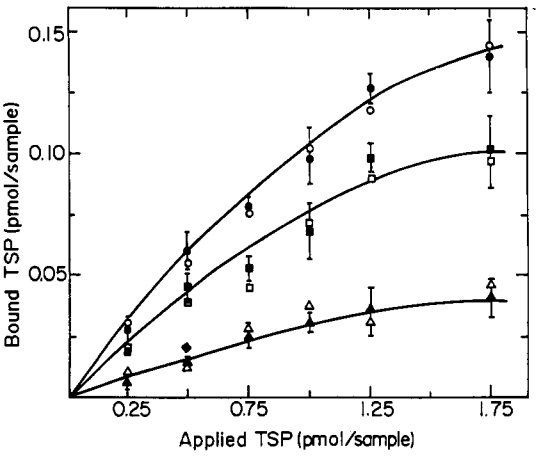

FIGURE 1: Dose dependence of binding of [^{125}I]EC-TSP by BAEC. The total binding of [^{125}I]EC-TSP to BAEC was determined by incubation of BAEC with increasing concentrations of [^{125}I]EC-TSP for 1 h at either 22 (●) or 4 °C (○). Nonspecific binding was determined in the presence of 10 μg/mL heparin at either 22 (▲) or 4 °C (△). The level of nonspecific binding obtained in the presence of 100 μg/mL heparin at 22 °C is shown by the solid diamond. Specific binding at 22 (■) and 4 °C (□) was determined by subtraction of the nonspecific binding values from the corresponding total binding values. See Materials and Methods for experimental details.

Table I: Stability of Labeled EC-TSP Incubated with Endothelial Cells, Expressed as the Ratio between Trichloroacetic Acid (TCA) Soluble and Insoluble Material[a]

incubation time (min)	TCA soluble material (cpm) / TCA precipitable material (cpm)
0	0.51 ± 0.07
15	0.49 ± 0.10
30	0.45 ± 0.14
60	0.60 ± 0.10

[a] Endothelial cells (2 × 10^5 cells/sample) were incubated with 1 μg/mL [^{125}I]EC-TSP in DMEM containing 0.2% BSA for various periods of time as indicated. Following incubation, the ratio between TCA soluble protein and TCA precipitable protein in the medium was determined according to Murphy-Ullrich & Mosher (1987).

In order to test whether degradation of [^{125}I]EC-TSP occurred following binding by the cells to an extent that could affect our binding studies, we measured trichloroacetic acid soluble radioactivity in the medium prior to and during the same time period of incubation (1 h) of the radiolabeled protein with the cells. No increase in trichloroacetic acid soluble radioactivity could be detected in the medium following a 1-h incubation of [^{125}I]EC-TSP with the cells (Table I), further corroborating our previous report (Lahav et al., 1987) that,

Table II: Inhibition of Binding of Intact TSP and of the 70-kDa Fragment to Endothelial Cells

incubation conditions[a]	% total binding[b] EC-TSP	70-kDa fragment
0.2% BSA	100	100
0.2% BSA + heparin (10 μg/mL)	32 ± 8	35 ± 9
0.2% BSA + fibronectin (100 μg/mL)	57 ± 4	42 ± 10
0.2% BSA + EC-TSP (100 μg/mL)	52 ± 10	46 ± 7
0.2% BSA + GRGDS (1 mM)	94 ± 2	not done
10% full serum	47 ± 10	32 ± 5
10% heparin-absorbed serum	87 ± 3	95 ± 10
anti-TSP antibody (1:10)[c]	35 ± 10	24 ± 12
hybridoma culture medium (1:10)[d]	95 ± 10	102 ± 11

[a] Endothelial cells (2×10^5 cells/sample) were incubated with either 1 μg/mL [^{125}I]EC-TSP or 1 μg/mL ^{125}I-labeled 70-kDa fragment for 1 h at 22 °C in DMEM containing various reagents as indicated. [b] The values shown represent average values ±SD of two experiments, each performed in duplicate. [c] 1:10 (vol/vol) of culture supernatant containing the monoclonal anti-TSP antibody (B_{7-3}) directed against the 70-kDa fragment. [d] 1:10 (vol/vol) of medium used for growing monoclonal antibody producing hybridomas. This was a control sample for testing the effect of the culture supernatant containing the monoclonal anti-TSP antibody.

in contrast to platelet TSP, EC-TSP was not degraded following binding by the cells. This lack of degradation is in accord with the evidence showing that EC-TSP is much more resistant to proteolysis than platelet TSP (Dardik & Lahav, 1987). Binding of [^{125}I]EC-TSP was significantly inhibited by unlabeled EC-TSP (Table II). Purified FN also markedly reduced EC-TSP binding (Table II). The inhibition of EC-TSP binding by FN was specific and could not be attributed to contaminating TSP, since FN was purified from human plasma by using a gelatin-Sepharose affinity column that does not bind TSP. The purity of the FN preparation was further confirmed by electrophoretic analysis (data not shown).

Binding of EC-TSP was reduced in the presence of 10% full serum as compared to that observed in the presence of 10% heparin-absorbed serum (depleted of FN and TSP as well as other heparin-binding components) (Table II). We found that while heparin-absorbed serum showed less than 10% inhibition of EC-TSP binding to endothelial cells relative to that found in DMEM containing 0.2% BSA, under the same conditions full serum reduced [^{125}I]EC-TSP total binding by ~50% as compared to that in 0.2% BSA (Table II). The inhibitory effect of full serum may be explained by the presence in it of bovine platelet TSP, suggesting that platelet TSP is able to compete with EC-TSP for binding to endothelial cells. However, the possible contribution of other heparin-binding compounds (e.g., FN, β-thromboglobulin, and platelet factor 4) cannot be excluded, since the noninhibiting depleted serum was prepared by passing the full serum over heparin-Sepharose.

EC-TSP binding to endothelial cells could not be inhibited by the cell-adhesion peptide GRGDS at the concentration of 1 mM (Table II). By contrast, under the same conditions binding of radiolabeled FN was inhibited by 70% as expected (data not shown).

The dissociation constant (K_d) obtained with EC-TSP is similar to that reported for platelet TSP binding to endothelial cells: 25 ± 1 nM for EC-TSP (Table I) as compared to 43 ± 30 nM for platelet TSP (Murphy-Ullrich & Mosher, 1987). However, the number of binding sites for EC-TSP is about 5 times higher than that found for platelet TSP: 2.6×10^6 sites/cell for EC-TSP (Table I) as compared to 5.1×10^5 sites/cell for platelet TSP (Murphy-Ullrich & Mosher, 1987). This observation may be explained by the rapid degradation (Murphy-Ullrich & Mosher, 1987) of platelet TSP following binding, which does not occur with EC-TSP.

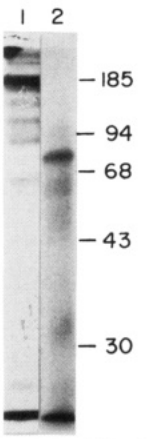

FIGURE 2: Electrophoretic analysis of the iodinated ligands used in cell-binding assays. Autoradiography of SDS–PAGE on 8% polyacrylamide gel of [^{125}I]EC-TSP (lane 1) and of the ^{125}I-labeled 70-kDa fragment (lane 2). The amount of radioactivity loaded in each lane was 100 000 cpm.

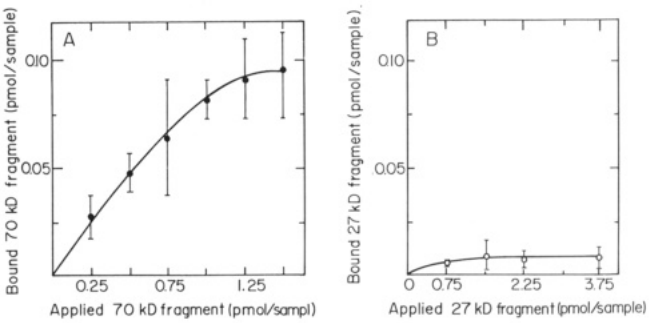

FIGURE 3: Binding of the ^{125}I-labeled 70-kDa fragment and the ^{35}S-labeled 27-kDa fragment by BAEC. Binding experiments were performed as described under Materials and Methods. (A) Dose dependence of binding of ^{125}I-labeled 70-kDa fragment to BAEC. The values shown represent specific binding determined as described in the legend to Figure 1. (B) Dose dependence of ^{35}S-labeled 27-kDa fragment binding to BAEC. The values shown represent specific binding determined as described in the legend to Figure 1.

Binding of the 70-kDa Chymotryptic Core Fragment of EC-TSP to Endothelial Cells. In order to identify the cell-binding domain on EC-TSP, we used radiolabeled chymotryptic fragments of EC-TSP as ligands in cell-binding assays. Metabolically labeled [^{35}S]EC-TSP was subjected to cleavage with chymotrypsin in the presence of EDTA for 5 min at 22 °C at a protease to protein ratio of 1:20. Two major fragments are produced under these conditions: the 27-kDa N-terminal fragment and the 70-kDa core fragment (Dardik & Lahav, 1987, 1989). Passing the digest over a heparin-Sepharose column resulted in binding of the 27-kDa fragment, which was subsequently eluted as described under Materials and Methods. Its purity was demonstrated earlier (Dardik & Lahav, 1989). The 70-kDa fragment, recovered in the effluent (Dardik & Lahav, 1989), was iodinated prior to its use in binding assays as described under Materials and Methods. The purity of the iodinated fragment is shown in Figure 2. Since the 27-kDa fragment can not be iodinated at high yield (Dardik & Lahav, 1987), it was used in its ^{35}S-labeled form.

As reported earlier, EC-TSP contains two heparin- and FN-binding domains for each ligand, one binding site resides on the 27-kDa fragment and the other on the 70-kDa fragment (Dardik & Lahav, 1987, 1989). Since binding of intact EC-TSP to endothelial cells is inhibited by both heparin and FN, we tested the 27-kDa as well as the 70-kDa fragment for cell-binding activity. We found that specific cell-binding

Table III: Analysis of Binding of [^{125}I]EC-TSP and ^{125}I-Labeled 70-kDa Fragment by Endothelial Cells

ligand	n^a	K_d (nM)	binding sites/cell × 10^{-6}
EC-TSP	3	25 ± 1	2.6 ± 0.6
70 kDa	7	47 ± 23	25.0 ± 1.5

^{a} n is the number of experiments performed.

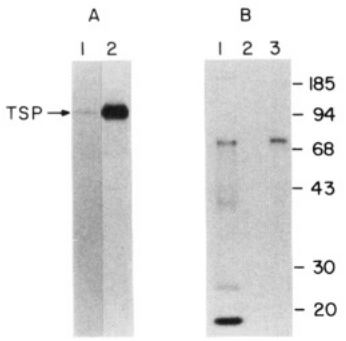

FIGURE 4: Radioimmunoprecipitation of the 70-kDa chymotryptic fragment by the monoclonal antibody B_{7-3}. (A) Radioimmunoprecipitation of [^{35}S]EC-TSP by hybridoma culture medium (lane 1) and by culture supernatant of the antibody B_{7-3} (lane 2). (B) Radioimmunoprecipitation of the chymotryptic digest of [^{35}S]EC-TSP produced in the presence of EDTA (lane 1) by hybridoma culture medium (lane 2) and culture supernatant of the antibody B_{7-3} (lane 3).

capacity was demonstrated only by the 70-kDa fragment and not by the 27-kDa fragment (Figure 3). The characteristics of binding of the 70-kDa fragment to endothelial cells were very similar to those of intact EC-TSP: binding of the ^{125}I-labeled 70-kDa fragment was dose dependent and saturable (Figure 3) and binding of the fragment could be inhibited to a similar extent as that of intact EC-TSP by unlabeled EC-TSP, as well as by FN, heparin, and full serum, but not by heparin-absorbed serum (Table II). By sharp contrast, binding of the 27-kDa fragment, even though significant, showed no dose dependence and was not inhibitable by TSP or by heparin at concentrations similar to those that inhibited binding of intact TSP or of the 70-kDa fragment. Thus, unlike the 70-kDa fragment, which demonstrated binding features very similar to those of intact EC-TSP, the 27-kDa fragment showed no specific cell-binding capacity (Figure 3).

The K_d of the interaction between the 70-kDa fragment and endothelial cells was calculated and found to be very similar to that found for intact TSP (47 ± 23 nM compared to 25 ± 1 nM; Table III). However, the number of binding sites for the 70-kDa fragment was somewhat higher than that for intact EC-TSP: (25.0 ± 1.5) × 10^6 sites/cell for the 70-kDa fragment as compared to (2.6 ± 0.6) × 10^6 sites/cell for intact EC-TSP (Table III). The observed difference in the number of binding sites is probably due to the difference in size between the 70-kDa fragment and the intact protein. The molecular masses of the trimeric forms of the fragment and intact TSP are 210 and 450 kDa, respectively. Unlike the 70-kDa trimer, the large molecule of intact TSP might interfere with ligand binding to adjacent receptors, when bound to the cell surface. Therefore, the number of receptors occupied at saturation by intact TSP may be smaller than that occupied by the 70-kDa fragment, leading to the observed difference in the number of binding sites.

Additional experiments were performed to further examine the specificity of the 70-kDa fragment binding to endothelial cells. First it was established that the monoclonal antibody B_{7-3} raised against human platelet TSP recognizes an epitope on the 70-kDa fragment of EC-TSP. This was done as follows:

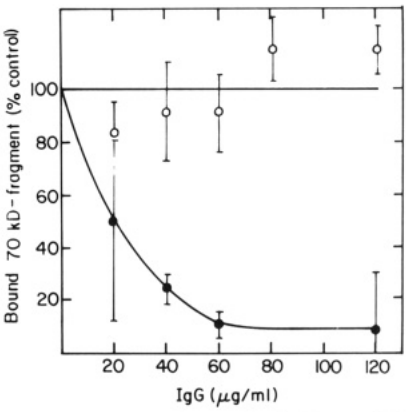

FIGURE 5: Effect of the anti-TSP antibody B_{7-3} on binding of the 70-kDa fragment to BAEC. A total of 200 μL of BAEC (4 × 10^4 cells) was incubated with 1 μg/mL ^{125}I-labeled 70-kDa fragment in the presence of increasing concentrations of an affinity-purified monoclonal anti-TSP IgG (B_{7-3}) (●) or normal mouse IgG (○). The values shown represent specific binding as percent of control specific binding obtained without the addition of any IgG. The experimental conditions are described under Materials and Methods.

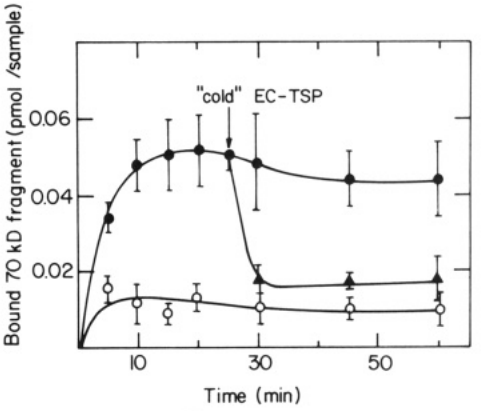

FIGURE 6: Displacement of ^{125}I-labeled 70-kDa fragment bound to BAEC by unlabeled EC-TSP. 200-μL aliquots of BAEC (4 × 10^4 cells) were incubated with 1 μg/mL ^{125}I-labeled 70-kDa fragment (1 pmol/sample) for varying periods of time at 22 °C in the absence (●) or presence (○) of 10 μg/mL heparin. After 25 min of incubation, three duplicate samples received 30 μL of unlabeled EC-TSP (▲) to a final concentration of 100 μg/mL (44 pmol/sample), and three other duplicate samples received 30 μL of Tris/NaCl (●). At various time points (30, 45, and 60 min), the cells were washed, and bound radioactivity was determined as described under Materials and Methods.

First it was shown that the antibody, in a precipitin complex (for details see Materials and Methods), specifically precipitates metabolically labeled EC-TSP (Figure 4A). Following chymotryptic digestion of EC-TSP, which yields two major fragments of 70- and 27-kDa (Figure 4B, lane 1), the antibody selectively precipitated the 70-kDa fragment but not the 27-kDa fragment (Figure 4B, lane 3). This precipitation was specific, as shown by the absence of precipitated material where an irrelevant antibody was attached to the precipitation complex prior to incubation with the fragment EC-TSP (Figure 4B, lane 2).

It was then shown that B_{7-3} inhibited the binding of the 70-kDa fragment to endothelial cells in a dose-dependent manner (Figure 5). In contrast, mouse serum IgG used as a control had no inhibitory effect when tested over the same range of concentrations (Figure 5), indicating specificity of the 70-kDa fragment binding. Further evidence for specificity was provided by the ability of unlabeled intact EC-TSP to displace the ^{125}I-labeled 70-kDa fragment, added after 25 min of incubation with the radioactive fragment (Figure 6). This observation also indicates that binding of the 70-kDa fragment

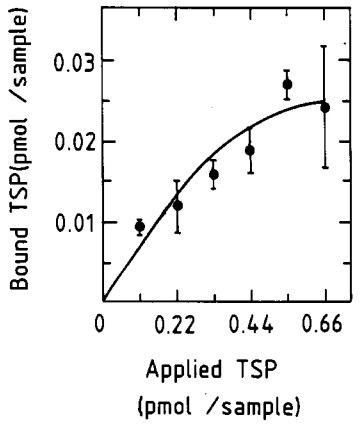

FIGURE 7: Dose dependence of [^{125}I]EC-TSP binding by normal granulosa cells. 200-μL aliquots of normal granulosa cells [(1–3) × 10^5 cells/sample] were incubated with increasing concentrations of [^{125}I]EC-TSP for 1 h at 22 °C. Following incubation, the samples were treated as described under Materials and Methods. The values shown represent specific binding determined by subtraction of the radioactivity bound in the presence of 50 μg/mL unlabeled EC-TSP (nonspecific binding) from the total cell-bound radioactivity.

Table IV: Analysis of Binding of [^{125}I]EC-TSP and ^{125}I-Labeled 70-kDa Fragment by Granulosa Cells

ligand	n^a	K_d (nM)	binding sites/cell × 10^{-6}
EC-TSP	2	1.8 ± 0.7	1.7 ± 0.4
70 kDa	2	38 ± 12	2.5 ± 0.5

[a] n is the number of experiments performed.

to endothelial cells is reversible and that the cells do not internalize it. Our results clearly demonstrate that the 70-kDa core fragment of EC-TSP, but not the 27-kDa N-terminal fragment, contains the cell-binding site. The observed similarity in the characteristics of binding between the 70-kDa fragment and the intact protein suggests that binding of EC-TSP to endothelial cells is mediated by the domain contained in this fragment.

EC-TSP Binding to Rat Granulosa Cells. Furman et al. (1986) have reported that extracellular matrix produced by corneal endothelial cells can regulate attachment and spreading, as well as the differentiation, of rat granulosa cells. The study of the interaction betwen granulosa cells and isolated extracellular matrix components has been limited, however, to fibronectin (Savion & Gospodarowicz, 1980; Skinner & Dorrington, 1984; Kim & Schomberg, 1989). Since TSP is a constituent of the matrix, we thought that granulosa cells might interact with EC-TSP. Indeed, we found that rat granulosa cells are capable of binding EC-TSP. Binding was specific, dose dependent, and saturable (Figure 7). The K_d is 1.8 ± 0.7 nM, and the number of binding sites is (1.7 ± 0.4) × 10^6 (Table IV). A series of experiments, similar to that performed with endothelial cells, demonstrated that EC-TSP binding to granulosa cells is inhibited by unlabeled TSP, FN, and the monoclonal anti-TSP antibody B_{7-3} but not by the GRGDS peptide (Table V). In contrast to the results obtained with endothelial cells, however, heparin had no inhibitory effect on EC-TSP binding to granulosa cells (Table V). Nonspecific binding was, therefore, determined in the presence of 50 μg/mL unlabeled EC-TSP.

Binding of the 70-kDa Fragment to Granulosa Cells. We next studied the ability of the 70-kDa core fragment to interact with granulosa cells. We found that the 70-kDa fragment indeed bound to granulosa cells, exhibiting binding features very similar to those of intact EC-TSP in terms of specificity,

Table V: Inhibition of Binding of Intact TSP and the 70-kDa Fragment to Granulosa Cells

inhibitor added[a]	% total binding[b] EC-TSP	% total binding[b] 70-kDa fragment
none	100	100
EC-TSP (50 μg/mL)	29 ± 1	26 ± 1
fibronectin (50 μg/mL)	33 ± 4	30 ± 15
anti-TSP antibody B_{7-3} (1:10)[c]	25 ± 8	32 ± 6
hybridoma culture medium (1:10)[d]	118 ± 23	95 ± 10
heparin (10 μg/mL)	110 ± 15	85 ± 5
GRGDS (0.1 mM)	92 ± 15	93 ± 12

[a] Granulosa cells (1 × 10^5) were incubated with either 0.5 μg/mL [^{125}I]EC-TSP or 0.5 μg/mL ^{125}I-labeled 70-kDa fragment for 1 h at 22 °C in DMEM + 0.2% BSA in the presence of various inhibitors as indicated. [b] The values shown represent average values ±SD of two experiments performed in duplicate. [c] 1:10 (vol/vol) of culture supernatant containing the monoclonal anti-TSP antibody (B_{7-3}) directed against the 70-kDa fragment. [d] 1:10 (vol/vol) of medium used for growing monoclonal antibody producing hybridomas. This was a control sample for testing the effect of the culture supernatant containing the monoclonal anti-TSP antibody.

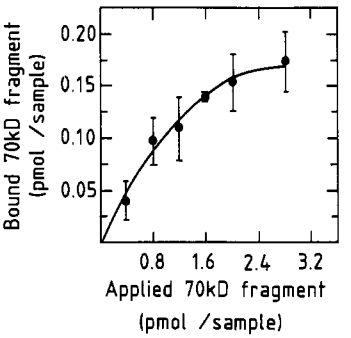

FIGURE 8: Dose dependence of ^{125}I-labeled 70-kDa fragment binding by normal granulosa cells. The experimental conditions were identical to those described in the legend to Figure 7 except for the ligand: ^{125}I-labeled 70-kDa fragment was used instead of [^{125}I]EC-TSP. The values show specific binding determined as in Figure 7.

dose dependence, saturability, and response to various inhibitors (Figure 8 and Table V). The affinity of granulosa cells for the 70-kDa fragment was markedly decreased as compared to that for intact TSP [K_d's of binding were 1.8 nM for EC-TSP and 38 nM for the 70-kDa fragment (Table 4)]. However, there was no significant difference between the intact molecule and its cell-binding domain in the number of binding sites per cell (1.7 × 10^5/cell for EC-TSP and 2.5 × 10^5/cell for the 70-kDa fragment).

These results demonstrate that, similarly to endothelial cells, granulosa cells can bind EC-TSP and that the binding site is also located in the 70-kDa core fragment. Unlike endothelial cells, which bound intact TSP and the 70-kDa fragment with similar affinity, granulosa cells demonstrated a 20-fold decrease in affinity upon TSP fragmentation. This observation suggests that the TSP receptor on granulosa cells is much more sensitive to structural modifications of TSP than the endothelial cell receptor.

Myoblasts Fail to Express Specific TSP Receptors. As shown in Table II, full serum at the concentration of 10% inhibited [^{125}I]EC-TSP binding to endothelial cells, whereas similar concentrations of serum depleted of FN and TSP did not, indicating that the cell-bound radioactivity detected in the presence of 10% full serum may be attributed mainly to nonspecific binding.

When we tested rat primary myoblasts for [^{125}I]EC-TSP binding, we found that binding was dose dependent but not saturable. Moreover, unlike endothelial cells, primary

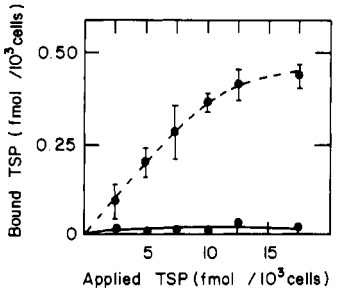

FIGURE 9: Binding of [^{125}I]EC-TSP to myoblasts. Dose dependence of binding of [^{125}I]EC-TSP to myoblasts (solid line) and to endothelial cells (dashed line) represented as the difference between the amount of ligand bound in the presence of 10% serum depleted of FN and TSP and that bound in the presence of 10% full serum.

myoblasts bound the same amount of radioactivity in the presence of 10% full serum and in the presence of 10% serum depleted of FN and TSP, indicating that no specific binding occurred (Figure 9).

Furthermore, detailed study showed that unlabeled EC-TSP, purified FN, and the monoclonal anti-TSP antibody B_{7-3} had no inhibitory effect on [^{125}I]EC-TSP binding to myoblasts, further supporting the nonspecificity of the interaction. Thus, although cell-associated radioactivity was detected following incubation of [^{125}I]EC-TSP with myoblasts, no evidence for specificity of binding could be obtained, indicating that myoblasts fail to express specific receptors for EC-TSP.

Discussion

We find that both endothelial cells and granulosa cells are capable of binding EC-TSP. Endothelial cells (McPherson et al., 1981), as well as granulosa cells (Dreyfus, Dardik, and Lahav, manuscript in preparation), synthesize and secrete TSP into the culture medium. By contrast, myoblasts that lack the capacity to synthesize TSP (Dreyfus & Lahav, 1988) fail to bind the protein. These results suggest that TSP synthesis by cells of a given cell type correlates with the ability of these cells to bind TSP. Additional evidence supporting this hypothesis is provided by the observation that there is a direct correlation between the ability of tumor cells to synthesize TSP and their binding capacity for both endogenous and exogenous TSP (Clezardin et al., 1989; Riser et al., 1988). Along the same line are the findings of Dreyfus and Lahav (1988), which indicate that the ability of extracellular matrix forming cells to incorporate TSP into the matrix also depends on their ability to produce TSP.

Using limited proteolytic fragmentation with chymotrypsin, we were able to identify the cell-binding domain of EC-TSP and to determine its affinity to endothelial and granulosa cells. Our results demonstrate that the cell-binding activity of EC-TSP is located in a domain contained in the 70-kDa fragment but not in the N-terminal heparin-binding domain. A recent report by Varani et al. (1988), showing that attachment of squamous carcinoma cells to TSP substrates is inhibited by the monoclonal anti-TSP antibody directed against the 70-kDa core fragment, but not by the antibody directed against the N-terminal heparin-binding fragment, further corroborates our contention that the cell-binding site is in the 70-kDa fragment. It is interesting to note that two reports addressing the question of the localization of the platelet-binding domain attribute it to the N-terminus (Gartner et al., 1984) and to the C-terminus (Galvin et al., 1985) but not to the 70-kDa core fragment. It is possible that there are different binding mechanisms that utilize different binding domains.

Several lines of evidence indicate that TSP can interact with cells at multiple sites and that the different TSP domains at

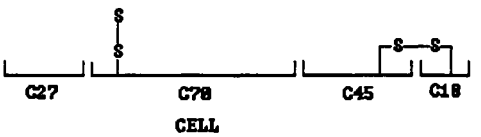

FIGURE 10: Location of the cell-binding domain of EC-TSP. This model shows the alignment of the proteolytic fragments of EC-TSP produced by chymotrypsin (C) and the location of the cell-binding domain. One EC-TSP polypeptide chain is shown; the molecular masses are indicated underneath each fragment.

which cell–TSP interactions occur mediate different processes. We have previously suggested that soluble TSP and surface-immobilized TSP comprise different entities in their interactions with the cells (Lahav et al., 1987). Indeed, more evidence in support of this hypothesis has been accumulating. In the case of smooth muscle cells, the monoclonal antibody D4.6 directed against the Ca^{2+}-sensitive region of TSP, at a concentration that inhibits the interaction of secreted TSP with the cell surface, fails to inhibit cell attachment to surface-immobilized TSP (Majack et al., 1988). This observation suggests that the expression of TSP on the smooth muscle cell surface may be mediated by a site on TSP distinct from the one involved in cell attachment to the immobilized protein. Moreover, there is evidence that a given cell type may possess different types of receptors for TSP, those that mediate binding of the soluble protein and those that mediate cell attachment to the surface-bound protein. Binding of soluble TSP to C32 melanoma cells is inhibited by the OKM5 antibody directed against the TSP receptor on the C32 cell surface (Asch et al., 1987). By contrast, the attachment of C32 cells to surface-immobilized TSP is not inhibited by the OKM5 antibody (Roberts et al., 1987). Attachment of endothelial cells to TSP-covered surfaces is inhibited by RGD-containing peptides (Lawler et al., 1988). In contrast, Taraboletti et al. (1990) showed that the GRGDS peptide significantly inhibits spreading but only slightly affects cell attachment of endothelial cells to TSP-coated plastic. Our results demonstrate that binding of soluble TSP to endothelial cells is not inhibited by the GRGDS peptide (Table II). RGD-containing peptides inhibit the attachment of U937 monocyte-like cells to plastic-bound TSP (Lawler et al., 1988) but not the interaction of these cells with TSP expressed on the surface of thrombin-activated platelets (Silverstein & Nachman, 1987). The latter example is even more striking, since it demonstrates different modes of recognition of two different forms of immobilized TSP. Thus different mechanisms of TSP recognition by a single cell type further support the existence of multiple cell-binding sites on TSP and, by them, distinction between immobilized and soluble TSP.

The cell-binding domain of EC-TSP contains, in addition, a high-affinity FN-binding site (Dardik & Lahav, 1989) and a low-affinity heparin-binding site (Dardik & Lahav, 1987) (Figure 10). Binding of the 70-kDa fragment to both endothelial and granulosa cells is inhibited by the same monoclonal anti-TSP antibody and by FN, suggesting that the two cell types share a common (or closely oriented) binding site on the TSP molecule, which is probably located near the FN-binding site of TSP. The inhibition of binding by FN may, thus, be explained by complex formation between FN and TSP (or the 70-kDa fragment), resulting in masking of the cell-binding site on TSP. As in the case of endothelial cells, TSP binding to granulosa cells was not affected by the GRGDS peptide, excluding the possibility of RGD-dependent recognition of TSP by these cells.

An interesting difference in binding characteristics between the two cell types was found, however, when the effect of

heparin on TSP binding was studied. Heparin inhibited binding of both intact TSP and of the 70-kDa fragment to endothelial cells but did not affect the binding to granulosa cells. The differential effect of heparin is likely to be due to the different nature of receptors on the two cell types rather than to the existence of different binding sites within the 70-kDa fragment. This difference is in accord with the different types of TSP receptors suggested by different groups. On endothelial cells, Murphy-Ullrich et al. (1987) have shown that the TSP receptor is heparin-like. Thus, it is possible that the inhibitory effect of heparin observed here is due to its competition with the heparin-like TSP receptor for the cell-binding site on the TSP molecule. This, however, would imply that the 27-kDa N-terminal heparin-binding fragment should also bind to endothelial cells. In our hands there was no specific binding of this fragment to endothelial cells. The reason for this discrepancy is not entirely clear and requires further investigation. We find that the binding characteristics of TSP to granulosa cells fit with the reported features of the OKM5 antigen, which was suggested as the TSP receptor of platelets (McGregor et al., 1989), monocytes, and two tumor cell lines (Asch et al., 1987). Binding of TSP to this receptor is not inhibited by heparin (Silverstein & Nachman, 1987), is RGD independent, and its structure significantly differs from that of cellular integrins which function via RGD recognition (Hynes, 1987). The function of the OKM5 antigen as the TSP receptor was recently questioned, however, by the findings of Oquendo et al. (1989), strongly suggesting the need for further study of this problem.

Lawler et al. (1988) have recently demonstrated that a GPIIb–IIIa-like integrin complex mediates RGD-dependent attachment of endothelial and smooth muscle cells to TSP. By contrast, C32 and G361 melanoma cells (Roberts et al., 1987) as well as squamous carcinoma cells (Varani et al., 1988) attach to TSP in an RGD-independent manner. Taken together, these observations further corroborate the existence of different types of receptors for TSP and indicate that different cell types use different mechanisms for binding TSP.

It is of great interest that different cell types differ not only in their mechanism of interaction with TSP but also in their ability to attach and spread on TSP substrate. TSP-covered surfaces fail to support adhesion of platelets (Lahav, 1988a) and endothelial cells (Lahav, 1988b; Lawler et al., 1988; Murphy-Ullrich et al., 1989). In contrast, substrate adhesion of some tumor cell lines is promoted by TSP (Varani et al., 1986; Roberts et al., 1987; Riser et al., 1988). Riser et al. (1988) have recently demonstrated that the ability of a given cell type to adhere on TSP depends on its capacity to synthesize and bind TSP. However, the synthetic and binding capacities are not sufficient to induce adhesive responsiveness to TSP in endothelial cells (Lahav, 1988b; Lawler et al., 1988; Murphy-Ullrich et al., 1989). In the granulosa cells, we also find that the attachment is inhibited by surface-immobilized TSP (Dardik, 1989), in spite of their ability to synthesize this protein (Dreyfus, Dardik, and Lahav, manuscript in preparation) and bind to the protein as shown in the work presented here. Thus, TSP binding by nontransformed cells may not be directly associated with TSP-supported substrate adhesion. Yet, the capacity of normal cells to bind TSP may be crucial for various important processes distinct from cell adhesion: a recent report demonstrates that cell-associated TSP is essential for smooth muscle cell proliferation (Majack et al., 1988). The regulation of TSP synthesis and matrix incorporation by cell density (Mumby et al., 1984), age (Kramer et al., 1985), growth factors (Majack et al., 1985), and exposure to heat shock (Ketis et al., 1988), as well as its ability to bind to cell surfaces and to modulate cell adhesion, imply a role for TSP in cell growth, migration, and development.

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