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ARTICLES

Heparin-Binding Domain, Type 1 and Type 2 Repeats of Thrombospondin Mediate Its Interaction With Human Breast Cancer Cells

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Abstract Thrombospondin is an adhesive glycoprotein that promotes breast cancer cell adhesion to human vascular endothelial cells (Incardona et al., 1995). In this study, we have identified the molecular domains of thrombospondin that mediate its binding to specific receptors on the human breast adenocarcinoma cell line, MDA-MB-231. Two recombinant fragments from the amino-terminus (TSPN18 and TSPN28), and the fusion proteins of the type 1 and type 2 repeats of human thrombospondin, inhibited binding of radiolabeled thrombospondin to MDA-MB-231 cells in suspension by 40–60% at 50 μg/ml whereas the type 3 repeat, carboxy-terminus and unfused glutathione-S-transferase as well as the synthetic peptide Gly-Arg-Gly-Asp-Ser (500 μg/ml) had little or no effect. Heparin and various glycosaminoglycans as heparan sulfate, chondroitin sulfates A, B or C, and fucoidan inhibited thrombospondin binding to MDA-MB-231 cells by more than 60% whereas dextran sulfate had only little effect. Treatment of cells with heparitinase, chondroitinase ABC, and hyaluronidase, but not with neuraminidase, induced 30–50% inhibition of thrombospondin binding suggesting the participation of both heparan sulfate and chondroitin sulfate cell surface-associated molecules. Inhibition of proteoglycan sulfation by chlorate or inhibition of glycosaminoglycan chain formation by two β-D-xylosides also led to a substantial inhibition of thrombospondin binding. Our results indicate that several domains within the thrombospondin molecule, namely the amino-terminus, type 1 and type 2 repeats, participate in its binding to specific receptors bearing sulfated glycosaminoglycans on MDA-MB-231 cells. Biological assays have indicated that, in addition to these domains, the peptide Gly-Arg-Gly-Asp-Ser inhibited MDA-MB-231 cell attachment to thrombospondin suggesting that the last type 3 repeat of the molecule may also contribute to its cell adhesive activity.

Key words: adhesion, breast cancer cells, thrombospondin, receptors, proteoglycans, heparin-binding peptides

Thrombospondin 1 (TSP) is a 420-kDa homotrimeric glycoprotein, originally found in blood platelets, which is synthesized and secreted by a wide variety of cells [Lawler and Hynes, 1986; Bornstein and Sage, 1994]. TSP can bind to several matrix constituents, including local growth factors, and to the cell surface [Lahav, 1993], and appears to play a role in a number of physiopathological processes such as platelet aggregation [Legrand et al., 1992], cell adhesion [Tuszynski et al., 1987a], proliferation [Majack et al., 1986; Castle et al., 1993], chemotaxis [Taraboletti et al., 1987], angiogenesis [Good et al., 1990], wound healing [Raugi et al.,

Abbreviations used: CSVTCG, Cys-Ser-Val-Thr-Cys-Gly; DMEM, Dulbecco's modified Eagle's medium; DX, cis/trans-decahydro-2-naphtyl-β-D-xyloside; GRGDS, Gly-Arg-Gly-Asp-Ser; GRGES, Gly-Arg-Gly-Glu-Ser; GST, glutathione-S-transferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide; NX, 2-naphtyl-β-D-xyloside; TSP, thrombospondin; TSPN18 and TSPN28, recombinant heparin-binding fragments of thrombospondin containing residues 1-174 and 1-248, respectively.

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1990], development [O'Shea and Dixit, 1988], differentiation [Castle et al., 1992], and apoptosis [Ren and Savill, 1995]. In addition, studies from several laboratories have led us to consider TSP as a molecule that may modulate tumor growth and metastasis [Tuszynski et al., 1987b; Castle et al., 1991].

Multiple TSP domains are involved in its interaction with various classes of cell surface receptors [Frazier, 1991; Adams and Lawler, 1993; Bornstein and Sage, 1994]. The amino-terminal domain that contains clusters of basic amino acid residues binds to heparan sulfate proteoglycans, such as syndecan, and sulfatides [Murphy-Ullrich et al., 1987; Roberts, 1988; Lawler et al., 1992]. The second and third type 1 repeats constitute major cell attachment sites. They contain a classic basic heparin-binding motif that acts cooperatively with two other sequences, namely the Trp-Ser-X-Trp and the Cys-Ser-Val-Thr-Cys-Gly (CSVTCG) sequence to bind to sulfated glycoconjugates [Prater et al., 1991; Guo et al., 1992]. The CSVTCG sequence was also shown to bind the transmembrane glycoprotein CD36 (Asch et al., 1992) and a receptor of 50/60 kDa [Tuszynski et al., 1993], both of them being expressed on certain tumor cells. The Arg-Gly-Asp sequence present in the last type 3 repeat may mediate TSP interactions with several integrins, namely $\alpha v\beta 3$ [Lawler et al., 1988], $\alpha IIb\beta 3$ [Tuszynski et al., 1989], $\alpha 2\beta 1$ [Tuszynski and Kowalska, 1991], $\alpha 4\beta 1$, and $\alpha 5\beta 1$ [Yabkowitz et al., 1993b]. Finally, the carboxy-terminal domain contains two cell attachment peptides which share the sequence Val-Val-Met and have been shown to interact with a 52 kDa receptor recently identified as the integrin-associated protein (IAP or CD47) [Gao et al., 1996]. A nonintegrin heterodimer of 80/105 kDa constitutes another receptor candidate for the carboxy-terminal domain of TSP [Yabkowitz et al., 1991]. Distinct receptors for TSP may be expressed on the same cell and act in a cooperative manner for cellular attachment [Asch et al., 1991; Guo et al., 1992]. Thus, the combination between the nature and number of specific TSP receptors gives the cell its unique "adhesive phenotype," as discussed recently by Adams and Lawler [1993].

In vitro studies have indicated that TSP is an adhesion factor [Tuszynski et al., 1987a; Asch et al., 1991; Yabkowitz and Dixit, 1991; Incardona et al., 1993] that promotes the motility [Taraboletti et al., 1987; Yabkowitz et al., 1993a] and proliferation [Castle et al., 1991] of carcinoma cells. Moreover during the critical step of extravasation, TSP may promote the formation of tumor cell aggregates and facilitate tumor cell anchorage to the endothelium [Clezardin et al., 1991; Incardona et al., 1995]. Recently however, evidence was provided for a negative role of TSP at least in certain cells or under certain circumstances as an increase in TSP expression was correlated with a reduction in malignant progression [Zajchowski et al., 1990; Weinstat-Saslow et al., 1994; Zabrenetzky et al., 1994] and TSP synthesis was shown to be positively controlled by tumor suppressor genes [Dameron et al., 1994; Zabrenetzky et al., 1994].

Clearly, more studies are needed to correlate TSP expression with tumor progression in a particular tissue as cell behavior, in terms of migratory or proliferative responses, will depend on the nature of the cell binding sites and subsequent intracellular signaling. In the present study, we have identified several domains within the TSP molecule, in particular heparin-binding domains, as potent inhibitors of TSP binding to the human breast adenocarcinoma MDA-MB-231 cell line that may be used to modulate the cell adhesive capacity.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 culture medium (F12) and phosphate-buffered saline (PBS) were obtained from GIBCO BRL (Cergy-Pontoise, France). Heat-inactivated fetal calf serum (FCS) was from Boehringer Mannheim (Meular, France). Heparin was supplied by Laboratoires Leo S.A. (Montigny-le Bretonneux, France). Carrier-free Na ^{125}I was purchased from Cis Biointernational (Gif-sur-Yvette, France). (^{2-3}H) glycine (19 Ci/mmol) was obtained from Amersham France (Les Ulis). The peptides Gly-Arg-Gly-Asp-Ser (GRGDS) and Gly-Arg-Gly-Glu-Ser (GRGES) were from Bachem Feinchemikalien AG (Budendorf, Switzerland). 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide (MTT) was from Sigma Chemicals Co (St. Louis, MO). 2-napthyl-β-D-xyloside (NX) and cis/trans-decahydro-2-napthyl-β-D-xyloside (DX) were obtained by a generous gift from T.A. Fritz and J.D. Esko, University of Alabama at Birmingham, USA. They were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 M and diluted in culture medium to the desired final concentration ($1–10^3$

μM). The final concentration of DMSO was 0.001% to 1% (v/v).

Glycosaminoglycans

Chondroitin sulfate A from bovine trachea, chondroitin sulfate B from bovine mucosa, chondroitin sulfate C from shark cartilage, heparan sulfate from bovine intestinal mucosa, dextran sulfate, and fucoidan from Fucus vesiculosus were purchased from Sigma.

Enzymes

Heparitinase from flavobacterium heparinum EC.4.2.2.8 was purchased from Seikagaku (Tokyo, Japan). Chondroitinase ABC from Proteus Vulgaris EC.4.2.2.4 was from ICN Pharmaceuticals (Orsay, France). Neuraminidase from Clostridium Perfringens EC.3.2.1.18 was from Boehringer Mannheim. Trypsin was purchased from GIBCO.

Thrombospondin

Thrombospondin 1 (TSP) was purified from the supernatant of thrombin-activated human platelets by heparin-Sepharose-4B chromatography followed by gel filtration, as described by Dubernard and Legrand (1991). The purified protein gave a single band (180 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions and was stored at −80°C in Tris-buffered saline, pH 7.4, containing 20% sucrose and 1 mM $CaCl_2$.

Recombinant Proteins

TSP1 cDNA clones were isolated by screening a bacteriophage λgt11 cDNA library prepared from human umbilical vein cells in culture (Clontech). The recombinant heparin-binding fragments from the amino-terminus of TSP, named TSPN18 and TSPN28, were expressed in E. coli strain A4255 under the control of the thermoinducible λP_L promoter and the CII ribosomal binding site as described [Legrand et al., 1994]. TSPN18 (18 kDa) and TSPN28 (28 kDa), contain residues 1–174 and 1–242 of human TSP1, respectively. The recombinant proteins were purified from inclusion bodies by chromatography on DEAE-Sepharose, CM-Sepharose, and heparin-Sepharose [Legrand et al., 1994].

Fusion proteins containing amino acid residues 385–522 (type 1 repeats), 559–669 (type 2 repeats), 784–932 (type 3 repeats), and 913–1152 (C-terminus) were obtained from human endothelial cell cDNAs as described [Legrand et al., 1992; Adams and Lawler, 1993]. Expression and purification of the fusion proteins and of unfused glutathione-S-transferase (GST) were carried out according to standard procedures [Smith and Johnson, 1988]. The purity of the fusion proteins was confirmed by SDS-PAGE.

Antibodies

Monoclonal antibodies (MAI and MAII) prepared against purified human platelet TSP or polyclonal antibodies prepared against the type 1, type 2, and type 3 fusion proteins (R3, R6 and R5) were prepared and characterized as previously described [Legrand et al., 1992]. These antibodies map different regions of the TSP molecule (Fig. 1).

Tumor Cell Lines

The human breast-adenocarcinoma cell lines MDA-MB-231 and MCF-7 were obtained from the ATCC (Rockville, MD). The cells were routinely grown in DMEM supplemented with 10% FCS, at 37°C in a humid atmosphere of 5% CO_2, and subcultured after detachment with 0.05% trypsin-0.02% EDTA. For binding experiments, cells grown to confluence were harvested by gentle detachment with 1 mM EDTA in PBS, pH 7.4, containing 1 g/l glucose, then washed three times and resuspended in DMEM containing 0.2% bovine serum albumin (BSA).

TSP Radiolabeling

Purified TSP was labeled with ^{125}I to a specific activity of 0.2–0.5 μCi/μg, using the chloramine-T method under mild conditions [Dubernard and Legrand, 1991]. Residual free iodine

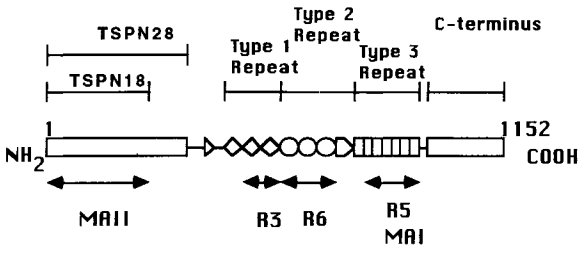

Fig. 1. Schematic representation of the TSP monomer, adapted from Lawler and Hynes (1986). The amino-terminus (NH2) is represented at the **left** and the carboxy-terminus (COOH) at the **right** of the figure. The recombinant peptides (TSPN18 and TSPN28) and fusion proteins (type 1, type 2, and type 3 repeats and C-terminus) are represented on the **upper part** and the antibodies directed against various regions of the molecule on the **lower part.**

was removed from the protein by gel filtration through a Sephadex G25 column (Pharmacia-LKB). The labeled protein was more than 97% precipitable by 20% trichloroacetic acid and appeared intact on SDS-PAGE followed by autoradiography [Dubernard and Legrand, 1991]. Aliquots were stored at −80°C in the presence of 20% sucrose.

Inhibition Assays of ^{125}I-TSP Binding to MDA-MB-231 Cells

Cells (1.2×10^5) in DMEM containing 0.2% BSA were aliquoted in Eppendorf tubes and centrifuged at 400*g* for 2 min. The pellets were *resuspended* in DMEM supplemented with 0.2% BSA and cells were incubated with 5 μg/ml ^{125}I-TSP, alone or in the presence of increasing concentrations of recombinant proteins (5–50 μg/ml) or various glycosaminoglycans (0.001 to 100 μg/ml), under gentle rotation. After 2 h at 4°C, 50 μl triplicate aliquots were centrifuged (400*g* for 4 min) through 450 μl of 20% sucrose in F12 medium supplemented with 0.2% BSA. The supernatants were removed by aspiration and the radioactivity associated with the cell pellets was determined in a gamma-counter (Beckman, San Ranon, CA). Nonspecific binding was routinely determined in the presence of 10 μg/ml heparin and subtracted from the total radioactivity in the cell pellets to yield the specifically bound radioactivity [Incardona et al., 1993].

Cell Enzymatic Treatments

Cells (1.2×10^5) in DMEM were incubated with increasing concentrations of trypsin (0.5 to 5.5×10^{-2} U/ml) for 15 min at 37°C. In another set of experiments, cells were pretreated with 10^{-6} to 10 U/ml heparitinase, chondroitinase ABC, neuraminidase, or hyaluronidase for 3 h at 37°C. At the end of the incubations, cells were pelleted, resuspended in DMEM containing 0.2% BSA, and processed for ^{125}I-TSP binding as described above.

Inhibition of Proteoglycan Biosynthesis

To reduce the degree of sulfation of cellular proteoglycans, MDA-MB-231 cells were grown in the presence of chlorate, a potent inhibitor of protein sulfation, as described [Baeuerle and Huttner, 1986]. As another approach, cells were grown in the presence of cis/trans-decahydro-2-naphtyl-β-D-xyloside (DX) or 2-naphtyl-β-D-xyloside (NX); these compounds inhibit chondroitin sulfate and heparan sulfate proteoglycan formation by competing with xylosylated core protein for glycosaminoglycan formation (Fritz et al., 1994). Both compounds prime chondroitin sulfate efficiently at concentrations ≥ 10 μM; NX begins to prime heparan sulfate at 10–30 μM whereas DX requires much higher concentrations to prime heparan sulfate [Fritz et al., 1994]. Briefly, MDA-MB-231 cells were washed with F12 medium containing a low amount of sulfate, and incubated with F12 medium supplemented with 0.2% BSA and 30 mM sodium chlorate or $1–10^3$ μM of either DX or NX β-D-xylosides. After 24 h at 37°C, cells were harvested with EDTA and processed for ^{125} I-TSP binding as described above. Chlorate and xylosides were maintained during the course of ^{125}I-TSP binding experiments. The results are expressed in percent inhibition of ^{125}I-TSP binding, considering as 100% the binding obtained when cells were incubated with 5 μg/ml of ^{125}I-TSP in F12 medium containing BSA alone (for chlorate effect) or BSA and DMSO (for xylosides effect). The influence of chlorate or xylosides on protein synthesis was investigated by adding 10 μCi/ml ^{3}H-glycine to the medium. After 24 h, the cells were washed, harvested with EDTA, sonicated, and DNA was quantitated following the method described by Labraca and Paigen [1980]. Quantitative analysis of cell protein synthesis was carried out by counting the radioactivity of samples containing equivalent quantities of DNA in a liquid scintillation analyzer (Packard-Tri-CARB 1600 TR, Downers Grove, IL).

Adhesion Assay

Wells of 96-well microtiter plates (Nunc-I-Immunoplate Maxisorp, Denmark) were coated with TSP (10 μg/ml) in acetate-buffered saline, pH 4.0, overnight at 4°C. Wells were then washed with DMEM containing 0.2% BSA and saturated with 2% BSA for 1 h at 37°C. At the end of the incubation, wells were overlaid with 100 μl of cells (8×10^4) in DMEM supplemented with 0.2% BSA and incubated at 37°C in a 5% CO_2 atmosphere. After 30 min, nonadherent cells were discarded, wells were washed once with PBS containing 0.2% BSA and incubated with 1 mg/ml MTT in PBS containing 0.2% BSA for 1 h at 37°C in a 5% CO_2 atmosphere, as described by Miller and McDevitt [1991]. Wells were then washed once with PBS and cells were solubilized

with 50 μl isobutanol before reading the optical density at 540 nm in a Titerteck Multiscan (ICN Biomedicals).

Inhibition of Cell Adhesion

MDA-MB-231 cells were incubated with soluble TSP or recombinant peptides (TSPN18 or TSPN28) or fusion proteins of the type 1, type 2, and type 3 repeats as well as the carboxy-terminus of TSP or with the synthetic peptides (GRGDS or GRGES) or with heparin for 1 h at 4°C. Cells were then seeded onto wells precoated with TSP and let to adhere as described above in the continual presence of the inhibitors. Each assay was run in quadruplicate. Nonspecific adhesion was determined with cells seeded onto wells precoated with 2% BSA and the value obtained was subtracted from that obtained with TSP-coated wells to yield the specifically adherent cells. Results are expressed in percent inhibition of cell adhesion considering as 100% the adhesion of cells seeded onto TSP in the absence of any treatment.

RESULTS

Effect of Heparin on ^{125}I-TSP Binding to Tumor Cells

As a first step to investigate the mechanism of TSP binding to breast cancer cells, we incubated suspensions of MDA-MB-231 and MCF-7 cells with 5 μg/ml ^{125}I-TSP for 2 h at 4°C and added heparin (10 μg/ml) under different experimental conditions (Fig. 2). When heparin was added simultaneously with ^{125}I-TSP (condition B), TSP binding was reduced by 60%, which is in agreement with our previous finding (Incardona et al., 1993). When cells were preincubated for 1 h with heparin (10 μg/ml), then washed and incubated with ^{125}I-TSP (condition C), no change in TSP binding was observed. In contrast, when cells were first incubated with ^{125}I-TSP, then washed and incubated for an additional hour with heparin (condition D), ^{125}I-TSP binding to tumor cells was reduced by 90%. These results demonstrate that the inhibitory effect of heparin results from a direct interaction with TSP, suggesting that TSP binds to heparin-like molecules on the surface of breast cancer cells.

Identification of Functional Domains Implicated in TSP Binding to Tumor Cells

In order to identify the molecular requirements for TSP binding to cell receptors, the binding of ^{125}I-TSP (5 μg/ml) to MDA-MB-231 cells was measured in the presence of increasing concentrations (5–50 μg/ml) of recombinant peptides or fusion proteins corresponding to different domains of the TSP molecule (see Fig. 1). As shown in Figure 3, recombinant proteins TSPN18 and TSPN28, as well as the fusion proteins corresponding to the type 1 and type 2 repeats of TSP inhibited ^{125}I-TSP binding in a dose-dependent manner with approximately 50% inhibition at 50 μg/ml. At this concentration, the unfused glutathione-S-transferase gave only a small (< 10%) inhibition whereas the fusion proteins corresponding to the type 3 repeat or C(carboxy)-terminus had no effect. The synthetic peptide GRGDS showed only a small inhibitory effect when used whereas the fusion proteins corresponding to the type 3 repeat or C(carboxy)-terminus had no effect. The synthetic peptide GRGDS showed only a small inhibitory effect when used at high (500 μg/ml) concentration whereas the control peptide GRGES had no effect. We have also used a panel of monoclonal and polyclonal antibodies reacting with various domains of TSP (see Fig. 1). However, we found that they all inhibited TSP binding to tumor cells suggesting that they may modify in a rather nonspecific manner the structure of the molecule (data not shown).

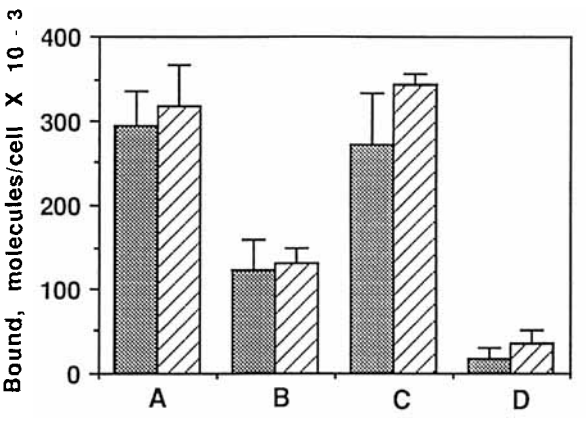

Fig. 2. Effect of heparin on ^{125}I-TSP binding to tumor cells. Binding of 5 μg/ml ^{125}I-TSP to 1.2 × 10^5 *MDA-MB-231 cells* (▨) or MCF-7 cells (▨), in DMEM containing 0.2% BSA, was determined as described in Materials and Methods in the absence **(A)** or in the presence of 10 μg/ml heparin **(B)**. In **(C)**, cells were incubated for 1 h with 10 μg/ml heparin, then washed and incubated with 5 μg/ml ^{125}I-TSP. In **(D)**, cells were incubated with 5 μg/ml ^{125}I-TSP, then washed and incubated for an additional hour with 10 μg/ml heparin. The results are expressed in molecules bound per cell × 10^{-3}, mean ± SD, n = 3.

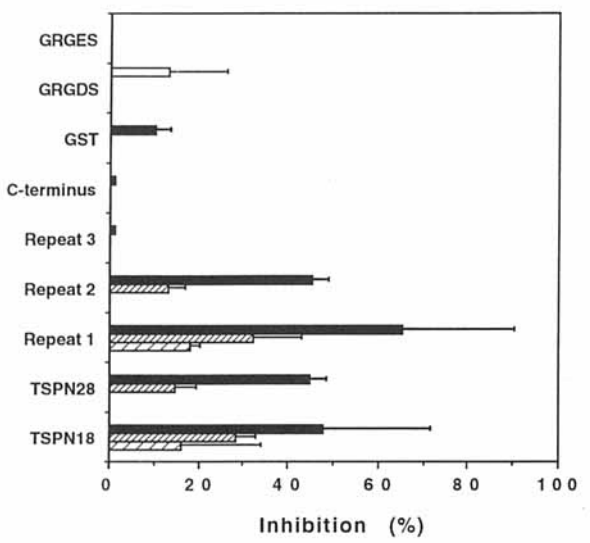

Fig. 3. Inhibition of ^{125}I-TSP binding to MDA-MB-231 cells by recombinant proteins. Binding of 5 μg/ml ^{125}I-TSP to MDA-MB-231 cells was measured in the presence of recombinant peptides TSPN18 or TSPN28, fusion proteins of the type 1, type 2, type 3 repeats, C-terminus or unfused glutathione-S-transferase (GST), used at 5 μg/ml (▨), 20 μg/ml (▨) or 50 μg/ml (■). Synthetic GRGDS or GRGES peptides were used at 500 μg/ml (□). Nonspecific binding was measured in the presence of 10 μg/ml heparin and subtracted from the total radioactivity in the cell pellet. The results are expressed as percentages of inhibition of ^{125}I-TSP binding, mean ± SD, n = 3.

Inhibition of ^{125}I-TSP Binding to MDA-MB-231 Cells by Various Glycosaminoglycans

The results obtained with heparin (Fig. 2) and with heparin-binding domains of TSP, both the amino-terminus and type 1 repeat (Fig. 3) suggested that a main receptor for TSP on breast cancer cells could be a heparan sulfate proteoglycan. We therefore tested the effects of various glycosaminoglycans, used in the range 0.005–100 μg/ml, on ^{125}I-TSP binding to MDA-MB-231 cells. As shown in Figure 4, heparan sulfate and various chondroitin sulfates behave as strong dose-dependent inhibitors of TSP binding giving more than 80% inhibition at high concentrations. Fucoidan and dextran sulfate were also inhibitory giving a maximum of 60% and 30% inhibition, respectively.

Enzymatic Pretreatment of Cells

Cells in suspension were treated with increasing concentrations (0.5 to 5.5 × 10^{-2} U/ml) of trypsin for 15 min at 37°C, washed, then incubated with 5 μg/ml ^{125}I-TSP. As a result of this treatment, TSP binding was decreased (Fig. 5A) up to 65% of initial binding at 5.5 × 10^{-2} U/ml of trypsin. Treatment of cells with increasing concentrations (10^{-6} to 10 U/ml) of heparitinase, chondroitinase ABC, or hyaluronidase for 3 h at 37°C also resulted in a gradual decrease of TSP binding up to 50–60% of initial binding at 10 U/ml (Fig. 5B). Neuraminidase was ineffective at all the concentrations tested.

Inhibition of Proteoglycan Biosynthesis

To further assess the role of sulfated macromolecular structures in TSP binding to cells, we inhibited sulfation of cellular glycosaminoglycans by incubating the cells for 24 h with sodium chlorate (30 mM) or with two synthetic β-D-xylosides, 2-napthyl-β-D-xyloside (NX), and cis/trans-decahydro-2-napthyl-β-D-xyloside (DX). These xylosides were used at different concentrations (1–10^3 μM) as they both prime chondroitin sulfate at concentrations ≥10 μM but NX requires lower doses than DX to prime heparan sulfate. As shown in Figure 6, the chlorate treatment inhibited binding of ^{125}I-TSP to tumor cells by 40%. The two xylosides tested inhibited TSP binding to cells in a dose-dependent manner. DX appeared to be somewhat more effective than NX and induced at 1 mM a similar inhibition (40%) to that observed with sodium chlorate. Because treatments with chlorate or β-D-xylosides were effected during the growth of the cells, we verified whether the treated cells had a similar protein synthesis as untreated cells. Cells were therefore grown in the presence of ^{3}H-glycine (10 μCi/ml) and the radioactivity associated with cell samples containing equivalent amounts of DNA was determined. No change in protein synthesis was observed with 30 mM sodium chlorate, or DX or NX up to 100 μM whereas 1 mM DX or NX inhibited protein synthesis by 50%.

Molecular Requirements for Tumor Cell Adhesion to a TSP Substratum

A quantitative cell adhesion assay was set up by incubating MDA-MB-231 cells with TSP-coated microwells as we previously described [Incardona et al., 1993]. The number of adherent cells was estimated by measuring the absorbance of the blue formazan product generated from the dye MTT as described [Miller and McDevitt, 1991]. The absorbance of the dye was linearly related to the quantity of cells added per microwell over the range 10^4–10^5 and began to

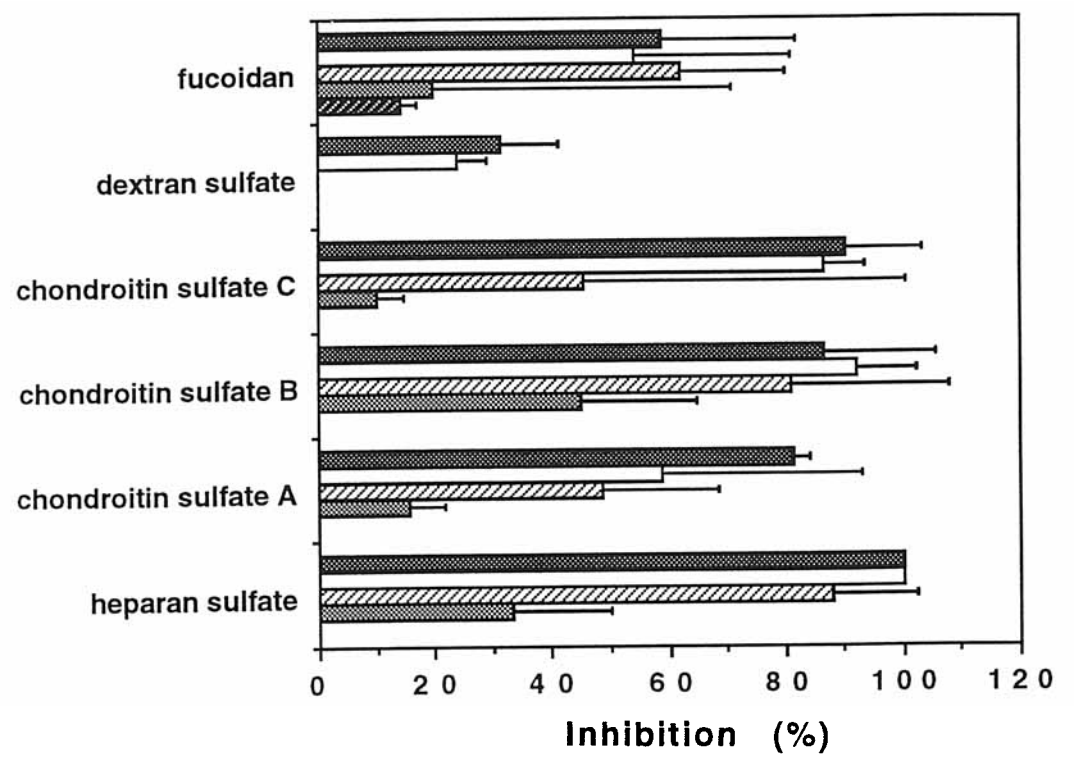

Fig. 4. Inhibition of ^{125}I-TSP binding to MDA-MB-231 cells by glycosaminoglycans. Cells were incubated with 5 μg/ml ^{125}I-TSP in the presence of 0.005 μg/ml (▩), 0.5 μg/ml (▩), 5 μg/ml (▨), 50 μg/ml (□) or 100 μg/ml (■) of various glycosaminoglycans. After 2 h at 4°C, cells were processed as described in Figure 2. The results are expressed as percentages of inhibition of ^{125}I-TSP binding, mean ± SD, n = 3.

reach a plateau at 1.5 × 10^5 seeded cells (not shown).

For inhibition assays, MDA-MB-231 cells were plated onto TSP-coated microwells at 8 × 10^4 cells/well for 30 min at 37°C. In order to determine the molecular characteristics of cell adhesion, tumor cells were preincubated with recombinant peptides (5–50 μg/ml), soluble TSP (20 μg/ml), or heparin (10 μg/ml). The synthetic peptides GRGDS and GRGES were used at 500 μg/ml. As shown in Figure 7, TSPN18, TSPN28, the type 1 and type 2 repeats, all inhibited in a dose-dependent manner the adhesion of tumor cells to TSP substratum whereas the type 3 repeat or C-terminus domain had no effect. The GRGDS peptide (1 mM) inhibited by 65% cell adhesion and, in spite of variable results obtained with the control GRGES peptide, this suggests that the Arg-Gly-Asp sequence of the TSP molecule may contribute to the adhesion of tumor cells. Preincubation of the cells with heparin (10 μg/ml) or soluble TSP (20 μg/ml) reduced cell adhesion by 80% and 60% respectively. Various antibodies to TSP also induced a dose-dependent decrease in cell adhesion when used at 5–50 μg/ml (data not shown).

DISCUSSION

Several studies have indicated a role for TSP in the complex process of breast cancer invasion and metastasis. Immunohistochemical studies have shown that TSP, primarily synthesized by myoepithelial cells and fibroblasts, accumulates in basement membrane surrounding in situ breast carcinoma and in the stroma of invasive ductal carcinoma [Wong et al., 1992; Clezardin et al., 1993; Tuszynski and Nicosia, 1994], where it may promote cell adhesion, motility, and proliferation [Tuszynski et al., 1987; Taraboletti et al., 1987; Incardona et al., 1993]. In addition, TSP is present in high levels in the blood of patients with breast cancer [Tuszynski et al., 1992] and may facilitate interactions of tumor cells with other vascular cells during the hematogenous dissemination [Clezardin et al., 1991; Incardona et al., 1995]. Recently however, the overexpression of TSP in human breast adenocarcinoma MDA-MB-435 cells transfected with

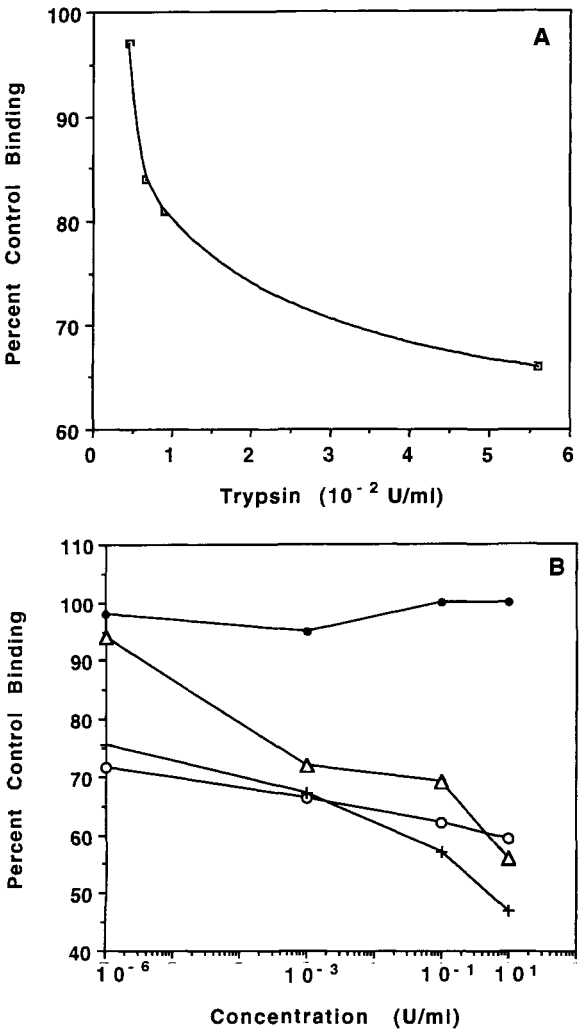

Fig. 5. Effects of enzymatic treatment on the ability of MDA-MB-231 cells to bind TSP. In **A**, cells (1.2×10^5) were treated at 37°C for 15 min with increasing concentrations of trypsin ($0.5–5.5 \times 10^{-2}$ U/ml). In **B**, cells were incubated at 37°C for 3 h with heparitinase (○), chondroitinase (△), neuraminidase (●) or hyaluronidase (+) at concentrations ranging from 10^{-6} to 10 U/ml. The results of an experiment are shown. Each point was made in triplicate and the mean value expressed as a percent of ^{125}I-TSP bound to untreated cells. Similar results were obtained in a second experiment.

a cDNA of TSP1 was shown to reduce in vivo primary tumor size, metastatic potential, and angiogenesis [Weinstat-Saslow et al., 1994].

The characterization of cell surface receptors for TSP may be of critical importance to understand its biological role in a particular neoplastic tissue. Among several putative TSP receptors, Tuszynski et al. [1994] localized the 50/60 kDa receptor, reacting with the CSVTCG sequence, in the epithelium of invasive ductal carcinoma as well as in cell extracts of the human BT-474 breast ductal carcinoma cell line. Clezardin et al. [1993] localized the αvβ3 receptor in most of the invasive lobular carcinoma cells and only 10% of invasive ductal adenocarcinoma cells whereas CD36 was found in a subpopulation of the invasive lobular carcinoma cells. In addition, based on an inhibition of TSP binding to MDA-MB-231 cells by heparin, we postulated the contribution of heparan sulfate glycosaminoglycans as TSP-binding molecules on these cells [Incardona et al., 1993]. In this study, we found that heparin could completely displace surface-associated TSP from MDA-MB-231 cells. Thus, if receptor sites were already occupied by endogenously synthesized TSP, we expected that preincubation of cells with heparin would result in an increased binding of radiolabeled TSP. Our results actually indicate that only a minority of sites on MDA-MB-231 cells are occupied by endogenous TSP. This is in relation to the low TSP synthesis by breast cancer cells as compared to stromal cells [Clezardin et al., 1993; Tuszynski and Nicosia, 1994] and confirms previous results that we obtained by flow cytometry analysis [Incardona et al., 1993, 1995]. In this study, we sought to identify the molecular domains of TSP involved in its binding to breast cancer cells by using recombinant fragments of the molecule as competitors of the binding of ^{125}I-TSP to MDA-MB-231 cells. The amino-terminus of TSP is a domain of major importance which contains a high affinity binding site for heparin [Lawler et al., 1992; Murphy-Ullrich and Mosher, 1987; Vogel et al., 1993]. Two recombinant heparin-binding fragments of 18 and 28 kDa (TSPN18 and TSPN28) produced a 50% inhibition of TSP binding, which was only slightly less than the inhibition obtained with heparin. Fusion proteins corresponding to the type 1 and type 2 repeats of TSP gave a similar inhibition whereas fusion proteins corresponding to the type 3 repeats and carboxy-terminus had no inhibitory effect. Thus our study underlines the role of the heparin-binding domains of TSP, both the amino-terminus and type 1 repeats, therefore suggesting the involvement of heparin-like molecules as major receptors for TSP on MDA-MB-231 cells. The observation that the type 2 repeats may also contribute to TSP binding to MDA-MB-231 cells is an intriguing result as no receptor reacting with this particular domain has been so far identified. The type 2 repeats are homologous to epidermal growth factor (EGF)

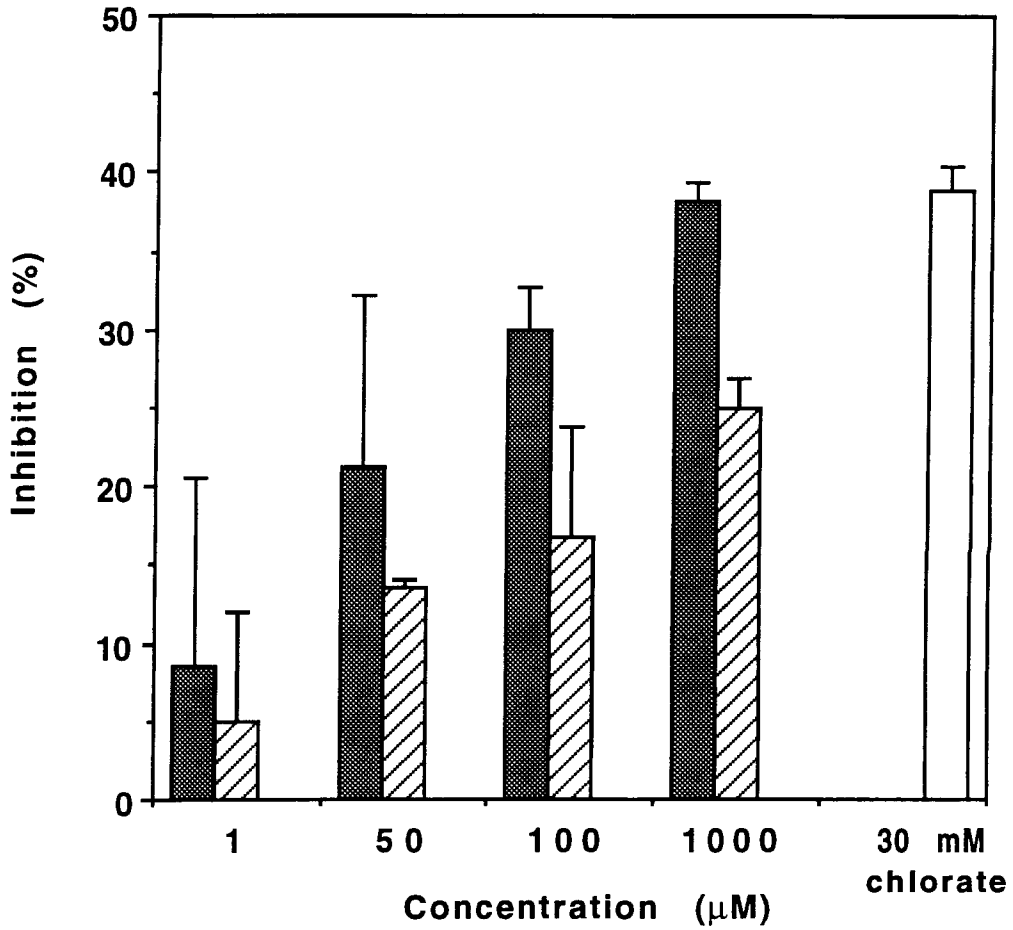

Fig. 6. Influence of proteoglycan sulfation on TSP binding to MDA-MB-231 cells. Cells were grown for 24 h in the presence of 30 mM sodium chlorate (□), or two different β-D-xylosides, DX (▩) or NX (▨), at concentrations ranging from 1 to 10^3 μM, as described in Materials and Methods. Chlorate and xylosides were maintained during the course of ^{125}I-TSP binding. The results are expressed as percentages of inhibition of ^{125}I-TSP binding, mean ± SD, n = 3.

and may be involved in the growth promoting effect of TSP [Engel, 1991]. There is no direct evidence however that these EGF-like sequences may react with EGF receptors on cells.

To further characterize the nature of cell surface-associated heparin-like molecules, we tested a panel of glycosaminoglycans for their ability to inhibit ^{125}I-TSP binding to MDA-MB-231 cells. Heparan sulfate, chondroitin sulfates A, B and C, and fucoidan all behave as strong inhibitors of TSP binding, whereas dextran sulfate displayed a much smaller effect. Furthermore, treatment of cells with heparitinase, chondroitinase ABC, or hyaluronidase also induced a strong decrease in TSP binding whereas neuraminidase had no effect. These results point to the participation of both heparan sulfate and chondroitin sulfate glycoconjugates as TSP-binding molecules on MDA-MB-231 cells and actually differ from those previously published with bovine aortic endothelial cells or human melanoma cells [Murphy-Ullrich and Mosher, 1987; Roberts, 1988], in which TSP was found to specifically bind to a heparan sulfate proteoglycan. The contribution of sulfated molecules in TSP binding to MDA-MB-231 cells was also demonstrated in experiments in which chlorate or β-D-xylosides were used to inhibit proteoglycan sulfation or proteoglycan formation, respectively. The observation that DX which prime preferentially chondroitin sulfate [Fritz et al., 1994] was as efficient, and even slightly more efficient than NX confirms the importance of chondroitin sulfate glycosaminoglycans as TSP-binding molecules. Taken together, these results lead us to propose that a major receptor for TSP on MDA-MB-231 cells might be syndecan, a proteoglycan which bears both a heparan sul-

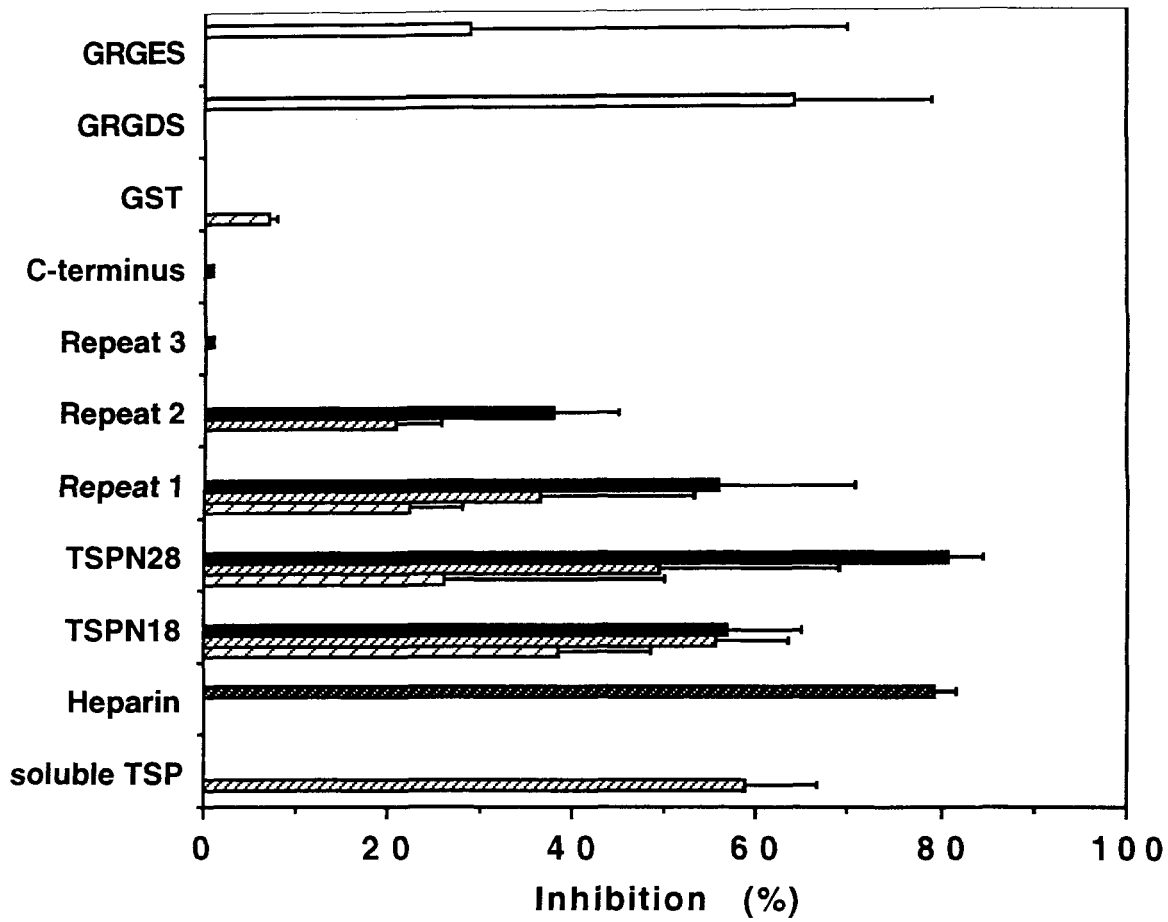

Fig. 7. Inhibition of MDA-MB-231 cell adhesion on TSP-coated substratum. The adhesion of MDA-MB-231 cells (8×10^4) to wells precoated with TSP (10 μg/ml) was measured in the presence of the recombinant peptides, TSPN18 or TSPN28, or fusion proteins of type 1, type 2, type 3 repeats or C-terminus, used at 5 μg/ml (▨), 20 μg/ml (▨) or 50 μg/ml (■), or with GRGDS and GRGES peptides used at 500 μg/ml (□), or with 20 μg/ml soluble TSP (▨) or 10 μg/ml heparin (■), as described in Materials and Methods. Results are expressed as percentages of inhibition of cell adhesion, mean ± SD, n = 3.

fate and chondroitin sulfate chain on its core protein [Bernfield and Sanderson, 1990].

The mechanism of breast cancer cell adhesion to a TSP substratum is probably complex as it involves additional binding sites to those required for the binding of soluble TSP. Thus, although we observed only minor inhibition of ^{125}I-TSP binding to MDA-MB-231 cells by the synthetic peptide GRGDS, we found that this peptide inhibited cell attachment to TSP adsorbed to plastic. This result underlines the critical importance of the conformation of matrix proteins in the regulation of their adhesive capacities, as discussed recently for TSP by Adams and Lawler [1993]. It is likely that the Arg-Gly-Asp sequence is cryptic in soluble TSP and its exposure is regulated by structural modifications of the molecule [Lawler et al., 1988; Sun et al., 1992] and, possibly, by its adsorption to a surface. Thus, in addition to heparan sulfate and chondroitin sulfate molecules, which probably represent major sites for TSP binding, an integrin receptor present on MDA-MB-231 cells may contribute to adhesion of these cells to matrix TSP. A likely candidate is αvβ3 as this integrin was shown to be expressed on lobular carcinomas [Clezardin et al., 1993]. Other integrins of the β1 family, namely α2-, α4- and/or α5-β1, which were shown to mediate the attachment of other cell types to TSP [Tuszynski et al., 1991; Yabkowitz et al., 1993] should also be considered as potential candidates.

In this study, we have identified the structural domains of TSP that mediates its binding to specific receptors on the human breast adenocarcinoma MDA-MB-231 cell line. Two heparin-binding domains of TSP, located within the amino-terminus and type 1 repeats, as well as

the adjacent EGF-like domains (type 2 repeats), were shown to contribute to the binding of soluble TSP to these cells. An additional site involving the Arg-Gly-Asp sequence located within the last type 3 repeat, adjacent to the carboxy-terminus, appeared to be involved in tumor cell attachment to a TSP substratum. Our findings confirm previous observations that multiple receptors on a same cell cooperate for cell adhesion to TSP [Adams and Lawler, 1993; Asch et al., 1991]. The identification of TSP receptors on human breast cancer cells, and related TSP binding sequences, may prove helpful to study how TSP affects signal transduction and cellular responsiveness and to better understand the role of TSP in neoplasia.

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