Identification and Characterization of a Tumor Cell Receptor for CSVTCG, a Thrombospondin Adhesive Domain

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Abstract. We have previously shown that peptides derived from the thrombospondin sequence, CSVTCG, promoted tumor cell adhesion. To further investigate this observation, the CSVTCG-tumor cell adhesion receptor from A549 human lung adenocarcinoma cells was isolated and characterized. A single protein peak was isolated by CSVTCG affinity chromatography which also analyzed as a single peak by anion exchange chromatography. The purified protein had a pI of 4.7 and analyzed on SDS-gels as a single band of $M_r = 50,000$ under nonreducing conditions and as two protein bands of $M_r = 50,000$, and 60,000 under reducing conditions. Purified CSVTCG binding protein (CBP) bound either CSVTCG- or TSP-Sepharose but showed little interaction with either VCTGSC- or BSA-Sepharose. CBP was cell surface exposed. CSVTCG derivatized with [^{125}I] Bolton-Hunter reagent was taken up by cells in a dose-dependent manner and the cell association was inhibited with a monospecific polyclonal anti-CBP antibody. Examination of the cell proteins crosslinked to labeled CSVTCG by SDS-gel electrophoresis revealed one band that comigrated with purified CPB. Using an in vitro binding assay, purified CBP bound mannose, galactose, and glucosamine-specific lectins. CBP bound TSP saturably and reversibly. The binding was Ca^{+2}/Mg^{+2} ion dependent and inhibited with fluid phase TSP and anti-CBP. Little or no binding was observed on BSA, fibronectin, GRGES, and GRGDS. Heparin, but not lactose, inhibited binding. Anti-CBP IgG and anti-CSVTCG peptide IgG inhibited A549 cell spreading and adhesion on TSP but not on fibronectin and laminin. These results indicate that CBP and the CSVTCG peptide domain of TSP can mediate TSP-promoted tumor cell adhesion.

THROMBOSPONDIN (TSP)[1] is a large multi-domain protein that was originally purified from platelets (15) but has since been found in many tissues including bone (23), muscle (39), skin (38, 39), and brain (20). A major physiological function of this protein appears to involve cell-extracellular matrix interaction because purified preparations of TSP promote cell-substratum interaction of a variety of cell types (34), including platelets (30, 33). Support for a role of TSP in matrix-cell interaction was demonstrated in a recent study by Arbeille et al. (3) who showed that TSP was localized in microfibrils at the junction between basement membrane and connective tissue in sections of human placenta, porcine arteries and skin. Additional support comes from several in vitro studies which show that TSP has a high binding affinity for components of the basement membrane such as collagen (8), fibronectin (13), and heparansulfate containing macromolecules (9).

Recently, our group (32) and two others (21, 22) independently demonstrated that peptides containing the sequence VTCG promoted cell attachment. Rich et al. (22) showed

that several peptides containing VTCG from region II of the circumsporozoite protein isolated from the malarial parasite *Plasmodium vivax* strongly promoted the attachment of T cells and myeloid cells. Prater et al. (21) showed that peptides containing VTCG and having sequences corresponding to amino acid residues 424–442 and 481–499 of TSP promoted G361 melanoma cell attachment. We demonstrated that peptides having the sequence CSVTCG and highly homologous analogues promoted the attachment of B16-F10 mouse melanoma, A549 human lung adenocarcinoma, bovine aortic endothelial cells and rabbit smooth muscle cells. These peptides also inhibited platelet aggregation and tumor cell metastasis presumably by competing with cell surface TSP receptors.

In this report we provide evidence that the anti-metastatic CSVTCG peptides bind tumor cell receptors that function as TSP tumor cell adhesion receptors. These receptors are distinct from known TSP receptors such as the integrins and may represent a new class of high affinity TSP receptors. These data also provide additional support for the hypothesis that TSP plays a major role in mediating tumor cell adhesive interactions.

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1. *Abbreviations used in this paper:* BCA, bicinchoninic acid; CBP, CSVTCG binding protein; TSP, thrombospondin.

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Materials and Methods

Materials

All reagents, unless specified otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO). Peptides were purchased from Multiple Peptide Systems (San Diego, CA). Tissue culture supplies, Na[^{125}I] and [^{125}I] Bolton-Hunter reagent were purchased from ICN Flow (Irvine, CA). Reagents for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA). Iodobeads and Bis(sulfosuccinimidyl) suberate (BS^3) were purchased from Pierce Chemical Co. (Rockford, IL). Microtiter plates, Removawell Immulon 4, were purchased from Fisher Scientific (Malvern, PA). CN-activated Sepharose and Phast System reagents were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ).

Cell Culture

Human A549 lung adenocarcinoma cells were cultured in DME containing 10% FCS, 100 units/ml penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin sulfate. Cells were grown to near confluency and were harvested in DME containing 0.02% EDTA. Cells were washed twice in Hepes buffered saline and the pellet either used immediately for purification of CSVTCG binding protein or stored frozen at $-70°C$ for future use.

Cell Adhesion Assay

Cell adhesion was performed by addition of cells suspended in DME (31) in an eight-well glass tissue culture slide spotted with various proteins (34). Anti-CSVTCG binding protein IgG (200 μg/ml) was added to the cell suspension, the cells were washed to remove excess antibody, and incubated for 1 h on BSA, FN, laminin or TSP-coated tissue culture plates. The cells were then aspirated, wells washed twice with PBS, the cells fixed, and adherent cells counted. To evaluate the effect of anti-CSVTCG peptide antibody, TSP-coated surfaces were treated with either 100 μg/ml of IgG or 100 μg/ml affinity purified anti-CSVTCG IgG in PBS for 30 min before addition of cells.

Direct ELISA

ELISA assays were performed as previously described (36). Briefly, microtiter plates were coated with 2 μg of protein either BSA, FN, TSP, or CSVTCG binding protein and blocked with 1% BSA for 1 h. Wells were incubated for 1 h with 50 μl of various dilutions of the first antibody in 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.05% Tween-20 (PBS-T). Wells were then washed three times in PBS-T and incubated for 1 h with 50 μl of a 1:800 dilution in PBS-T of alkaline phosphatase coupled rabbit anti-goat IgG. Wells were washed three times with PBS-T followed with three washes of PBS-T buffer containing no Tween-20 and treated with 50 μl of alkaline phosphatase substrate solution (1 mg/ml of p-nitrophenylphosphate in 0.10 M glycine, pH 10.4, containing 1 mM ZnCl$_2$ and 1 mM MgCl$_2$). After 30 min, color development was stopped by the addition of 5 μl of 1 N NaOH and absorbances determined at 405 nm.

Antibodies

CSVTCG was coupled to maleimide activated keyhole limpet hemocyanin (KLH) according to the instructions provided with the Pierce Imject Activated Immunogen Conjugation Kit, Pierce Chemical Co. Approximately 4 mg of peptide was coupled to 2 mg of KLH. A goat was injected subcutaneously with 1 mg of peptide-protein conjugate suspended in complete Freund's and boosted with two additional injections of 500 μg of peptide-protein conjugate in incomplete Freund's adjuvant three and 5 wk after the first injection. Goat immunization and collection of serum was accomplished commercially through Hazelton Research Products, Inc. (Denver, PA).

Polyclonal anti-CSVTCG was affinity purified on TSP-Sepharose. Briefly, IgG prepared by ammonium sulfate precipitation of 100 ml of antisera was dissolved in 50 ml of Hepes-buffered saline, pH 7.35 (column buffer), and passed over a 1 ml column of Sepharose containing 2 mg of coupled TSP. The TSP column was then washed with 10 ml of column buffer and eluted with 0.1 M sodium citrate buffer, pH 2.7. One ml fractions were collected in tubes containing 50 μl of 1 M Tris base in order to neutralize the final solution. Protein fractions were pooled, concentrated and assessed for anti-TSP activity by ELISA analysis.

Polyclonal CSVTCG binding protein antiserum was raised in a rabbit after four 50 μg injections every 3–4 wk. The first injection was given with complete Freund's adjuvant and subsequent injections were administered with incomplete Freund's adjuvant. Rabbit immunization and collection of

serum was accomplished commercially through Hazelton Research Products, Inc. Antibody titers and specificity were determined by ELISA.

TSP Purification

TSP was purified from Ca^{+2} ionophore A23187-activated human platelets as previously described (37).

Protein Assays

Protein concentrations were determined by the bicinchoninic acid protein assay, adapted for microtiter plates, as described by Pierce Chemical Co. (Rockford, IL). BSA was used as standard.

Gel Electrophoresis

SDS-PAGE was performed using the Pharmacia Phast Gel or Biorad mini-slab gel systems. Protein samples were reduced with 5% beta-mercaptoethanol. Gels were silver stained, dried, and autoradiograms were prepared from the dried gels using intensifying screens (DuPont Cronex Lighting Plus screens mounted in Spectroline Cassettes, Reliance X-Ray Inc., Oreland, PA). Kodak X-Omat-AR film (Eastman Kodak Co., Rochester, NY) was used and developed according to the instructions provided with the film. Films were exposed for varying lengths of time at $-70°C$.

Protein and Cell Labeling

Purified CSVTCG-binding protein was labeled with [^{125}I]iodine using Iodobeads as previously described (10). Briefly, 12 μg of purified protein dissolved in 100 μl of octylglucoside buffer was incubated with one Iodobead for 10 min. Unreacted iodide was removed on a small column of Sephadex G-25 equilibrated in octylglucoside buffer as previously described (29). The specific activity of protein obtained in a typical experiment was 0.5–2.0 × 10^4 cpm/ng.

A549 cells were surface labeled with [^{125}I]iodine using lactoperoxidase (29). Briefly, cells from a 75-mm flask containing a near confluent monolayer of cells were harvested, washed, and suspended in 0.5 ml of DME containing 0.17 units/ml lactoperoxidase and 500 uCi of [^{125}I]-iodide. A 1 μl aliquot of 0.002% H$_2$O$_2$ in PBS was added with gentle mixing every minute for 4 min. The iodination was then stopped by the addition of 600 μl of 2 mM NaI in PBS, cells washed twice in PBS, and the pellet solubilized in NP-40 detergent as described below for purification of the CSVTCG binding protein.

Preparation of Coupled-Sepharose Gels for Specificity Experiments

Approximately 4 mg of either CSVTCG or VCTGSC or 2 mg of TSP or BSA were coupled to 1 ml of CN-activated Sepharose according to the instructions provided by Pharmacia LKB Biotechnology, Inc. Gels were equilibrated in 20 mM Tris HCl, pH 7.0, containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 5 mM octyl glucoside before use.

Preparation of [^{125}I]-Bolton-Hunter Derivatized CSVTCG

A 100 μl aliquot of 10 mg/ml solution of CSVTCG in PBS was added to 1 mCi of Bolton-Hunter reagent previously sparged with nitrogen to remove benzene as indicated in the instructions provided with the Bolton-Hunter kit. The reaction mixture was incubated with occasional shaking for 1 h at 4°C. A 100 μl aliquot of a 1 mg/ml glycine solution was then added to quench any unreacted reagent. The final reaction mixture was chromatographed on a 2-ml column of Sephadex G10 equilibrated in PBS containing 0.1% gelatin. The void volume peak (400 μl containing 4.1 × 10^8 cpm) was collected and stored under nitrogen at 4°C until needed.

Cell Association and Cross-linking of [^{125}I]-Bolton-Hunter Derivatized CSVTCG

A549 cells were grown to near confluency in 6 well tissue culture plates. The media was then removed and cells rinsed twice with serum-free DME. Wells were treated with 1 ml serum-free media containing varying amounts of peptide (5 × 10^6 cpm/ml in a typical experiment) for 1 h at 37°C. Monolayers were washed twice with PBS, harvested with EDTA and counted to determine cell-associated CSVTCG. For cross-linking, the washed monolayers were treated with 0.5 ml of a 2 mM BS^3 solution in

PBS for 10 min at room temperature, excess cross-linker removed, and the monolayer extracted with 100 μl of SDS gel sample buffer containing 4 M urea, 1 mM EDTA, 1 mM PMSF, and 100 μl leupeptin. The extracted cell layer was then analyzed by SDS-gel electrophoresis followed by autoradiography.

Purification of CSVTCG Binding Proteins

CSVTCG binding proteins were purified by affinity chromatography from A549 detergent extracts. Briefly, approximately 4.0×10^7 A549 cells were harvested with EDTA and the pellet washed once with DME. The cell pellet was then homogenized on ice with 5 ml of solubilization buffer (10 mM Tris HCl, pH 7.5, containing 0.5% NP-40, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM leupeptin, 1 mM phenylmethyl sulphonyl fluoride). The cell lysate was then centrifuged at 4°C for 20 min at 100,000 g to remove undissolved material. The supernatant was passed three times over a 5 ml column containing 4 mg of CSVTCG coupled to 1 ml of CN-activated Sepharose equilibrated in Hepes buffered saline, pH 7.35. The unbound material was washed with 20 ml of column buffer (10 mM Tris HCl, pH 7.5, containing 0.05% NP-40, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM leupeptin, 1 mM phenylmethyl sulphonyl fluoride). Bound material was eluted with elution buffer (0.1 M Tris, pH 10.2, containing 0.05% NP-40, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM leupeptin, 1 mM phenylmethylsulphonyl fluoride) by collecting 1 ml fractions in a tube containing 70 μl of 1 N HCl to neutralize the final solution. The proteins eluting in the first 10 fractions were applied to an anion exchange column (Mono Q, Pharmacia LKB Biotechnology, Inc.) equilibrated in anion exchange column buffer (20 mM Tris HCl, pH 8.0, containing 5 mM octyl glucoside). The bound material was eluted at 0.7 ml/min with a 10 ml gradient of NaCl (100% 1 M NaCl) and the column monitored at 280 and 260 nM. The bound material routinely began to elute at 0.3 M NaCl and the gradient was held to allow the proteins to elute isocratically. The eluted fractions were concentrated and analyzed for TSP binding activity and further characterized by SDS-gel electrophoresis and isoelectric focusing.

Binding Assay

[^{125}I]-labeled CSVTCG binding protein and unlabeled CSVTCG binding protein were combined in binding buffer (20 mM Tris buffered saline, pH 7.35, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% NP-40) such that the final concentration was 1.8 μg/ml and 100 μl of solution contained ~200,000 cpm. Microtiter wells were coated with proteins and peptides by drying under nitrogen a 50 μl aliquot of a 40 μg/ml solution of protein or peptide in 20 mM bis-tris-propane buffer, pH 6.5. Wells were then treated with 200 μl of 1% BSA for 1 h, washed once with binding buffer, and incubated with 100 μl of [^{125}I]-labeled CSVTCG binding protein for 60 min. Well were then washed three times by aspiration with 200 μl of binding buffer and bound radioactivity determined by counting each well in a gamma counter.

Western Immunoblotting

Anti-CSTSCG antibody was characterized by Western immunoblotting using Pharmacia's Phast electrophoresis system. Whole cell extracts were prepared by dissolving 4×10^5 cells in 100 μl of SDS sample buffer. Approximately, 100 ng of protein was separated under reducing conditions on an 8-25% polyacrylamide gradient SDS gel. Gels were either silver stained or the proteins were electrophoretically transferred onto nitrocellulose paper. The paper was then blocked with 3% BSA in PBS-T and treated for 1 h with either 1.37 μg/ml of affinity purified goat anti-CSVTCG or 50 μg/ml anti-CSVTCG binding protein IgG for 1 h, washed with PBS-T, developed according to the instructions provided with the VECTASTAIN ABC immunoperoxidase system, (Vector Laboratories, Burlingame, CA). Controls were the respective control IgG fractions.

Results

Purification of CSVTCG Binding Protein

Approximately, 4.0×10^7 A549 cells were grown to near confluency and harvested with EDTA. The washed pellet was solubilized with NP-40 detergent and the extract applied to a CSVTCG-Sepharose column. One protein peak was eluted at high pH in the first 10 fractions as detected by SDS-gel
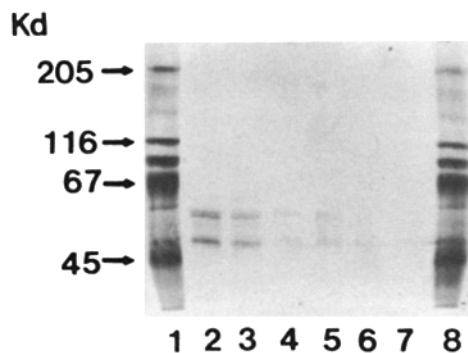

Figure 1. SDS-gel analysis of the high pH eluate from the CSVTCG affinity column. Detergent extracts of A549 cells were adsorbed on CSVTCG-Sepharose, unbound proteins washed, and bound proteins eluted at high pH. Fractions were analyzed under reducing conditions by SDS-gel electrophoresis on a 7.5% polyacrylamide gel and visualized by silver staining. Lanes *1* and *8*, mol standards; lanes *2-7*, column fractions 1, 3, 5, 7, 9, and 10, respectively.

electrophoresis under reducing conditions (Fig. 1). This material could be adsorbed to an anion exchange column at pH 8 and eluted with 0.30 M NaCl as a single homogenous peak having a high ratio of absorbance at 260 to 280 nm (Fig. 2). The purified material analyzed on SDS-gel electrophoresis as two polypeptide bands of $M_r = 60,000$ and $50,000$ under reducing conditions and one polypeptide of $M_r = 50,000$ under nonreducing conditions (Fig. 3). Analysis by isoelectric focusing revealed that the purified material resolved as one band with a pI in the range of 5.00–4.65 (Fig. 4). Approximately, 100 μg of protein was recovered from 4 $\times$ 10^7 cells.

Specificity of the Interaction of CSVTCG with the CSVTCG Binding Protein

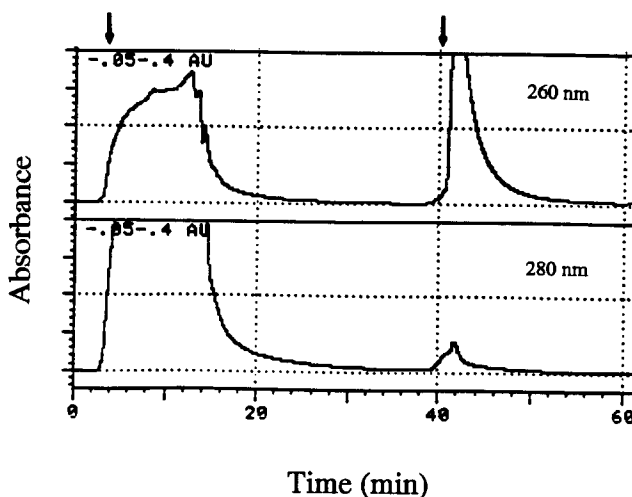To determine if the CSVTCG binding protein binds CSV-

Figure 2. The anion exchange elution profile of CSVTCG binding protein. The high pH eluate from the CSVTCG affinity column was applied to a Mono Q anion exchange column and eluted with 0.3 M NaCl as described in Materials and Methods. The column was monitored at 260 and 280 nm. The first arrow at the top of the chromatogram indicates when the sample was applied and the second arrow shows when the 0.3 M NaCl was applied to elute the adsorbed proteins.
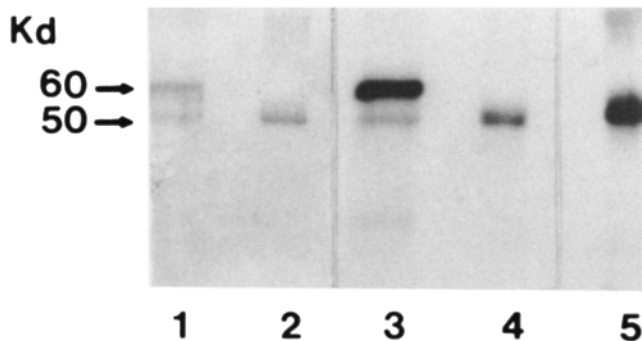
Figure 3. SDS-gel analysis of the CSVTCG-binding proteins. Detergent extracts of A549 cells and [^{125}I]iodine surface labeled A549 cells were adsorbed on a CSVTCG column, the column washed with column buffer, and then eluted with elution buffer, pH 10.2 or with 0.1 M citrate buffer, pH 3.0. The nonradioactive sample was further chromatographed on anion exchange chromatography as described in Materials and Methods. The non-radioactive sample was then labeled with [^{125}I]iodine. Lanes *1* and *2*, Coomassie blue stained and labeled CSVTCG binding protein; lanes *3* and *4*, autoradiogram of lanes *1* and *2*; lane *5*, autoradiogram of CSVTCG binding protein isolated from [^{125}I] surface labeled A549 cells. Samples in lanes *1* and *3* were reduced with 5% β mercaptoethanol. Samples in lanes *2*, *4*, and *5* were nonreduced. The gel was 8% polyacrylamide.

TCG specifically two experiments were performed. In the first experiment, [^{125}I]-labeled CSVTCG binding protein was evaluated for its capacity to bind and be eluted from Sepharose derivatized with either TSP, BSA, CSVTCG, or VCTGSC. CSVTCG binding protein could only be bound
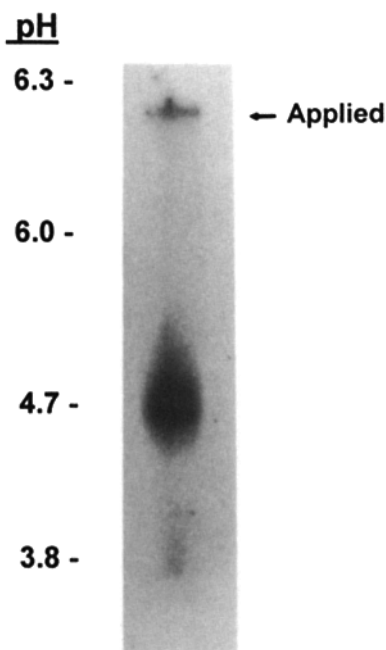

Figure 4. Isoelectric focusing analysis of [^{125}I]CSVTCG binding protein. [^{125}I]CSVTCG binding protein (100,000 cpm) was analyzed on a pH 2.5–6.5 isoelectric focusing gel. The arrow indicates where the sample was applied on the gel and the small amount of material remaining at the point of application represents nonspecifically adsorbed material. Similar amounts of material were adsorbed to the point of application when the sample was applied at different places on the gel but the major band always focused at pH 4.7 (data not shown).
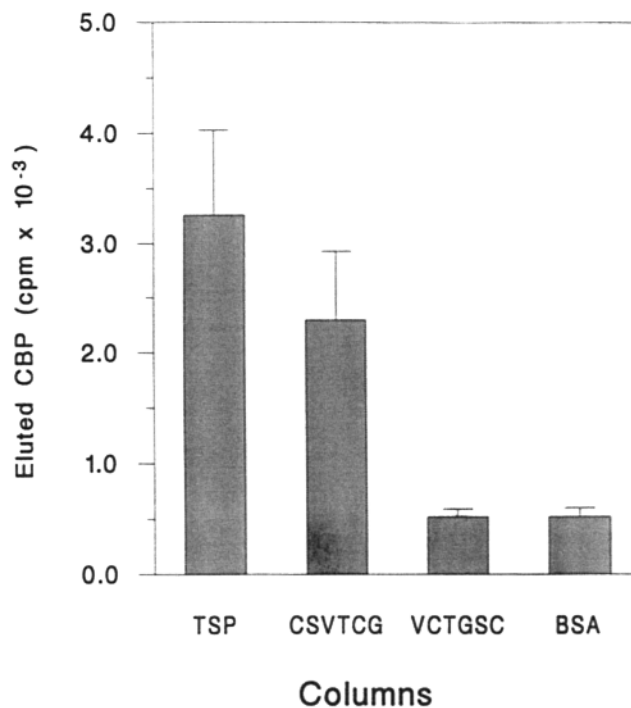

Figure 5. Interaction of [^{125}I]CSVTCG binding protein with Sepharose coupled to various peptides and proteins. 100 μl slurries of coupled-Sepharose derivatized with either TSP, BSA, CSVTCG, or VCTGSC as described in Materials and Methods were equilibrated in 20 mM Tris HCl, pH 7.0, containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ and 5 mM octyl glucoside (equilibration buffer). To each resin 1 × 10^6 cpm of [^{125}I]CSVTCG binding protein in equilibration buffer was added with mixing and incubated for 1 h at room temperature. The resins were gently centrifuged to remove excess liquid, and washed twice with 200 μl of equilibration buffer followed by two more washes of equilibration buffer containing 0.5 M NaCl. Resins were then eluted with 200 μl of 0.1 M citrate buffer, pH 3.0, containing 0.05% NP-40 detergent. The amount of eluted radioactivity is depicted in the bar graph. Each value is the average of four separate resins and the error bars represent the standard deviation.

and eluted from TSP and CSVTCG-coupled resins (Fig. 5). In the second experiment, CSVTCG derivatized with [^{125}I]-Bolton-Hunter reagent was added to monolayers of A549 cells in the presence and absence of anti-CSVTCG antibody and cross-linked with a water soluble cross-linking agent (Fig. 6). In the absence of antibody, CSVTCG became cell-associated in a dose-dependent manner, whereas in the presence of antibody cell-association was nearly completely inhibited (*top inset*, Fig. 6). Cross-linking of the derivatized CSVTCG to cells identified one major band that comigrated with the purified CSVTCG binding protein on SDS-gels (*bottom inset*, Fig. 6). These experiments indicate that the CSVTCG binding protein recognizes CSVTCG both in vitro and on the cell surface.

Characterization of CSVTCG Binding Protein

To characterize the CSVTCG binding protein, the protein was labeled with [^{125}I]iodine and evaluated for its capacity to bind TSP. Analysis of the labeled material by SDS-gel electrophoresis followed by autoradiography revealed that the M_r = 60,000 polypeptide was predominantly labeled (Fig. 3). The labeled CSVTCG binding protein bound TSP in a time-dependent manner which became time-indepen-
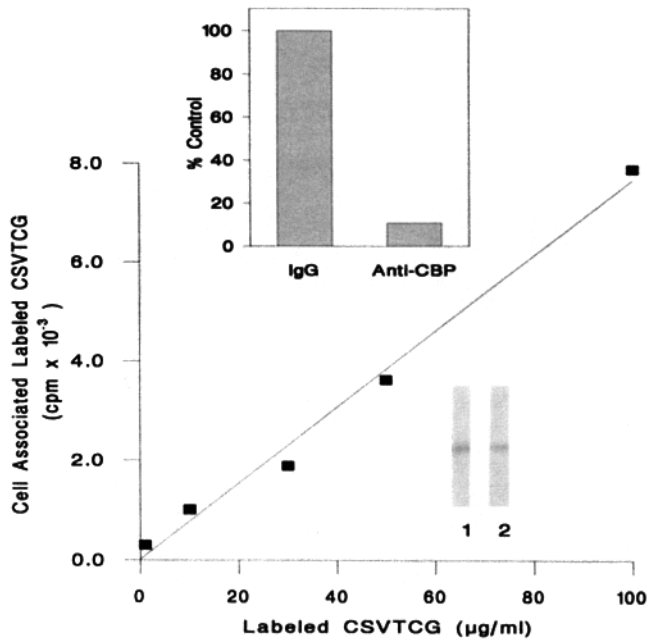
Figure 6. Interaction of [^{125}I]CSVTCG with A549 cells. Cells were treated with [^{125}I]CSVTCG and the amount of cell-associated peptide was measured as described in Materials and Methods. The top inset shows the amount of cell-associated peptide when cells were incubated with 10 μg/ml of labeled peptide in the presence of either 100 μg/ml rabbit IgG or 100 μg/ml rabbit anti-CSVTCG binding protein (anti-CBP). The bottom inset shows an autoradiogram of an SDS-gel of purified CSVTCG binding protein and total cell extract cross-linked with BS^3 as described in Materials and Methods. Proteins were separated on an 8% gel under nonreducing conditions. Lane *1*, purified [^{125}I]CSVTCG binding protein. Lane *2*, total cell extract from cells cross-linked in the presence of [^{125}I]CSVTCG.

Figure 7. The divalent ion requirements for the interaction of CSVTCG binding protein with TSP. CSVTCG binding protein was radiolabeled with [^{125}I]iodine and binding to immobilized TSP was performed as described in Materials and Methods. Concentrations of all salts and EDTA were 1 mM. The designations Ca, Mn, Mg are $CaCl_2$, $MnCl_2$, and $MgCl_2$, respectively.

The Effect of Anti-CSVTCG Binding Protein and Anti-CSVTCG Peptide Antibody on Tumor Cell Adhesion

To investigate the importance of the CSVTCG-binding protein in mechanisms of TSP-promoted cell substratum adhesion, a polyclonal CSVTCG binding protein antiserum was raised in a rabbit and a polyclonal CSVTCG antiserum was

dent after 60 min (data not shown). The binding was maximal in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ or 1 mM $CaCl_2$ alone, whereas 1 mM $MgCl_2$ and 1 mM $MnCl_2$ supported significantly less binding (Fig. 7). Binding (55% of maximal) was also observed in the presence of 1 mM EDTA (Fig. 7). The CSVTCG binding protein had the greatest binding affinity for TSP whereas fibronectin, BSA, and the peptides GRGES and GRGDS had negligible binding activity (Fig. 8). The CSVTCG binding protein bound TSP saturably with an apparent binding constant of 0.30 μM. The binding constant was estimated from the least squares fit of the data to a rectangular hyperbola (correlation coefficient of 0.994) assuming M_r = 50,000 (Fig. 9). This binding was reversible because increasing concentrations of unlabeled protein added to the incubation mixture caused a dose-dependent inhibition of binding (Fig. 9). Incubation of CSVTCG binding protein with TSP and heparin in the fluid phase resulted in a 64 and 80% inhibition of binding respectively, whereas BSA and lactose had no effect (Fig. 10). These results indicate that the CSVTCG-binding protein can interact specifically with TSP in vitro and that binding may be mediated by CSVTCG-binding associated glycoconjugates. Consistent with this conclusion is the observation that the CSVTCG-binding protein contains carbohydrate. We found that the purified protein bound galactose-, mannose-, and galactosamine-specific lectins (Table I).
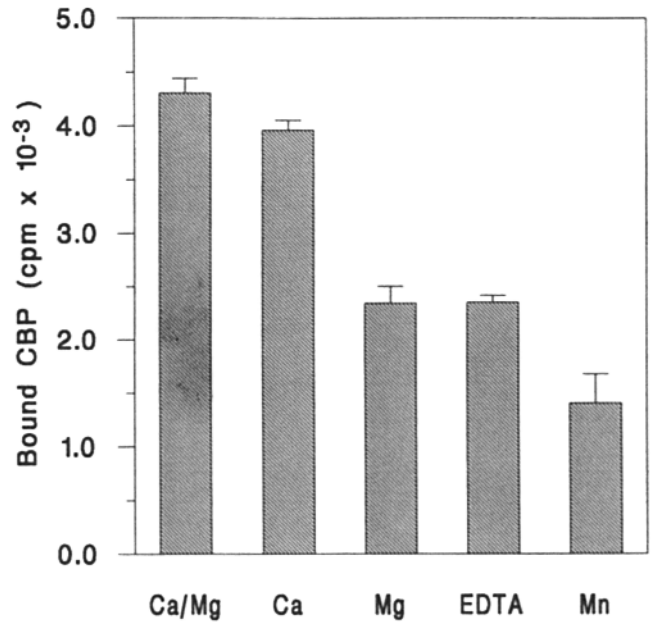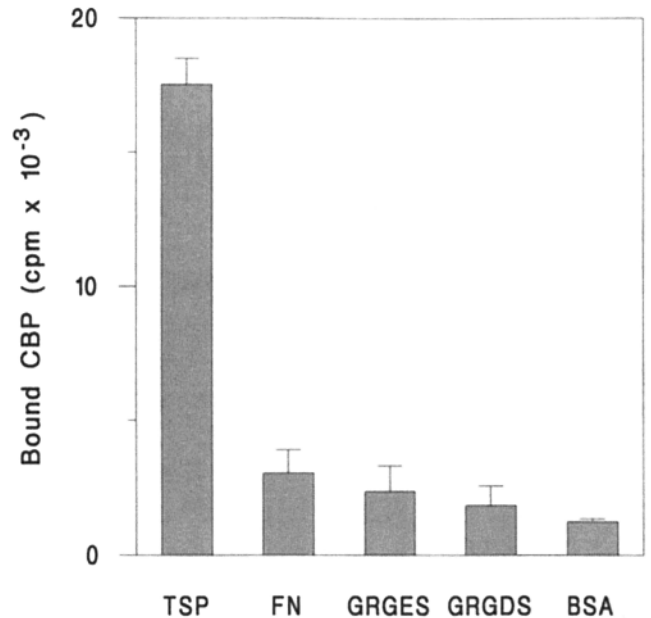

Figure 8. The specificity of the interaction of CSVTCG binding protein with TSP. CSVTCG binding protein was radiolabeled with [^{125}I]iodine and binding to the various immobilized proteins and peptides was performed as described in Materials and Methods.
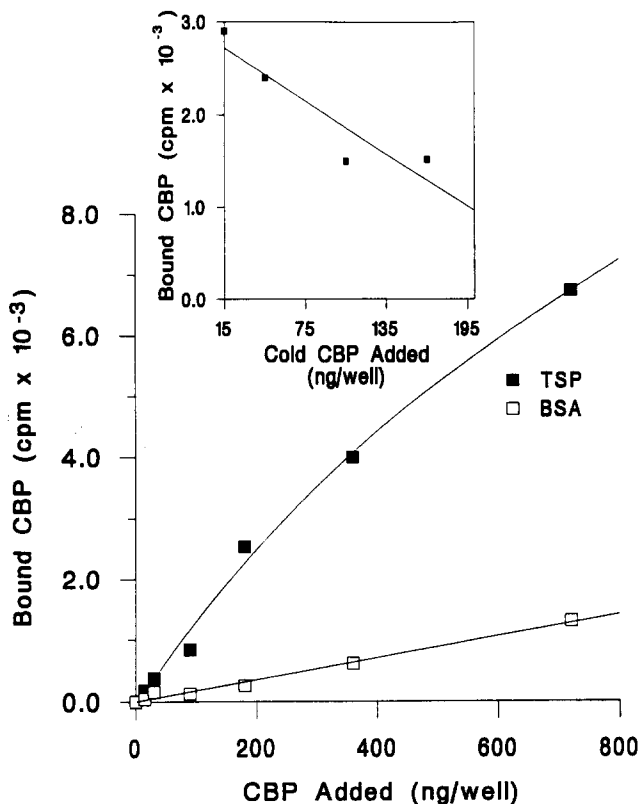
Figure 9. Concentration dependence of the binding of ^{125}I-labeled CSVTCG binding protein to TSP. Binding was performed to immobilized TSP in binding buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ as described in Materials and Methods. The closed squares show specific binding to TSP and the open squares show binding to BSA. Specific binding is defined as cpm bound to TSP minus cpm bound to BSA. Values given are the mean of two duplicate determinations and are representative of a typical experiment. Duplicate values differed by no more than 5%. The line drawn was computer generated by a least squares fit to a rectangular hyperbola with a calculated dissociation constant of 0.30 μM (GraphPad Inplot, Version 3.1, San Diego, CA). The inset shows binding performed as above except that the ratio of unlabeled to labeled CSVTCG binding protein was progressively increased.

raised in a goat. The antibodies were monospecific as determined by direct ELISA (data not shown) and by immunoblot analysis (Fig. 11). Anti-CSVTCG binding protein antibody specifically recognized the M_r = 60,000 subunit of purified CSVTCG binding protein and A549 cell extracts under reducing conditions (Fig. 11 *A*). Anti-CSVTCG recognized the 180-kD subunit of TSP and a minor 160-kD degradation product (Fig. 11 *B*). Both anti-CSVTCG and anti-CSVTCG binding protein IgG fractions almost completely inhibited A549 cell adhesion to TSP-coated surfaces but had no effect on cell adhesion to fibronectin or laminin (Fig. 12). In addition, both anti-CSVTCG and anti-CSVTCG binding protein IgG nearly totally prevented cell spreading on TSP but had no effect on FN and laminin-mediated cell spreading (data not shown). These results strongly suggest that the CSV-TCG-binding protein functions as a TSP cell adhesion receptor and the TSP sequence, CSVTCG, functions as a tumor cell adhesive domain of TSP. Consistent with this interpretation is the cell surface orientation of the CSVTCG-binding protein which could be labeled with [125I]iodine on intact cells (Fig. 3).
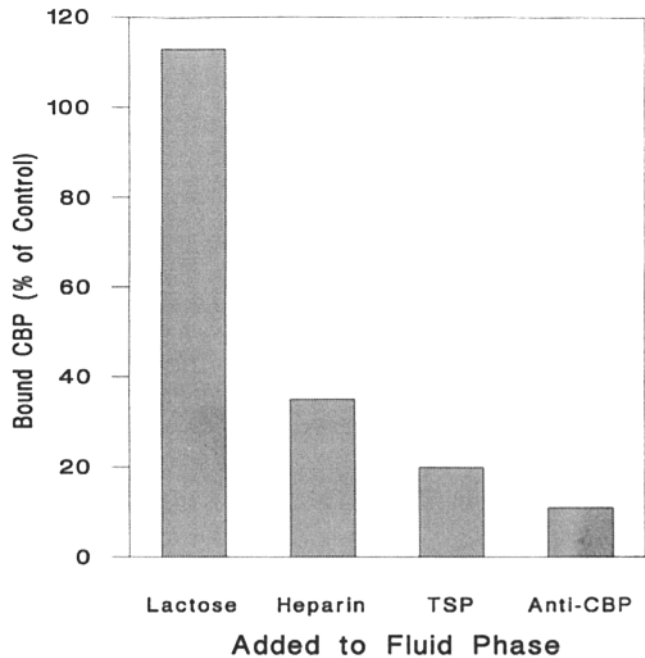

Figure 10. The effect of fluid-phase TSP, IgG, lactose, heparin, and anti-CSVTCG binding protein (anti-CBP) on the binding of CSVTCG binding protein to immobilized TSP. Binding was performed in binding buffer containing either 10 μg/ml BSA, 10 μg/ml TSP, 100 μg/ml lactose, 100 μg/ml heparin, 100 μg/ml rabbit IgG, or 100 μg/ml anti-CBP. The 100% control for lactose, heparin and TSP was the amount bound in the presence of fluid phase BSA. The 100% control for anti-CBP was the amount bound in the presence of rabbit IgG. Values given were calculated from the mean of two duplicate determinations and are representative of a typical experiment. Duplicate values differed by no more than 5%.

Discussion

A major focus of our work has been to show that TSP plays a significant role in the pathogenesis of tumor spread. Several lines of evidence both from our laboratory and from the work of others strongly suggest that TSP may mediate one or more steps in the metastatic cascade. We observed that mice injected with tumor cells together with TSP developed 2–3 times more lung metastases than did controls (27). A likely explanation for these results is that TSP promoted the

Table I. Lectin-CSVTCG Binding Protein Interactions

Lectin	Bound cpm	Sugar specificity
Concanavalin A	1263	alpha-D-man glucose
Lens culinaris	896	alpha-D-man
Lycopersicon esculentum	2140	(D-glcNAc)₃
Ptilota plumosa	1112	alpha-D-gal
Dolichos biflorus	379	alpha-D-galNAc
Tetragonolobus purpureas	641	alpha-L-fuc

Lectins were coated in microtiter wells by incubating each well with 100 μl of a 40 μg/ml solution of lectin in PBS. Wells were washed twice in PBS, blocked with BSA, and incubated with 50 μl of [125I]CSVTCG binding protein in binding buffer (130,000 cpm). Wells were washed twice in PBS and bound radioactivity counted. The amount bound to BSA-coated plates which accounted for <9% of the observed radioactivity was subtracted from each value given in the table. Values are the average of three determinations which varied by <5%.
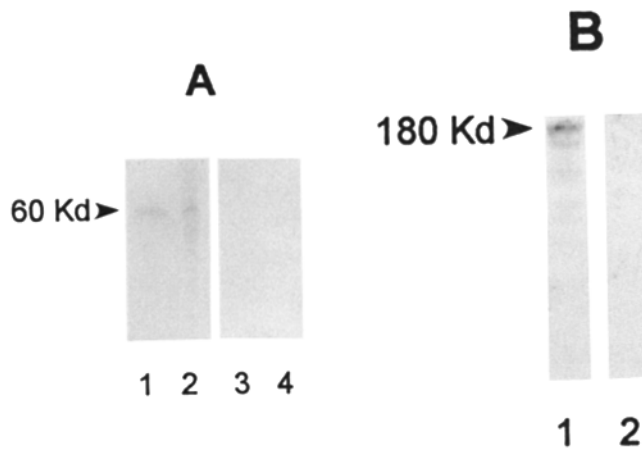
Figure 11. Characterization of anti-CSVTCG binding protein and anti-CSVTCG by immunoblotting. Proteins were analyzed on an 8-25% gradient gel under reducing conditions as described in Materials and Methods. (*A*) Anti-CSVTCG binding protein blot of purified CSVTCG binding protein (lane *1*), SDS extract of A549 whole cells (lane *2*), IgG blot of CSVTCG binding protein (lane *3*), IgG blot of A549 cells (lane *4*). (*B*) Anti-CSVTCG blot of purified TSP (lane *1*), IgG blot of TSP (lane *2*).
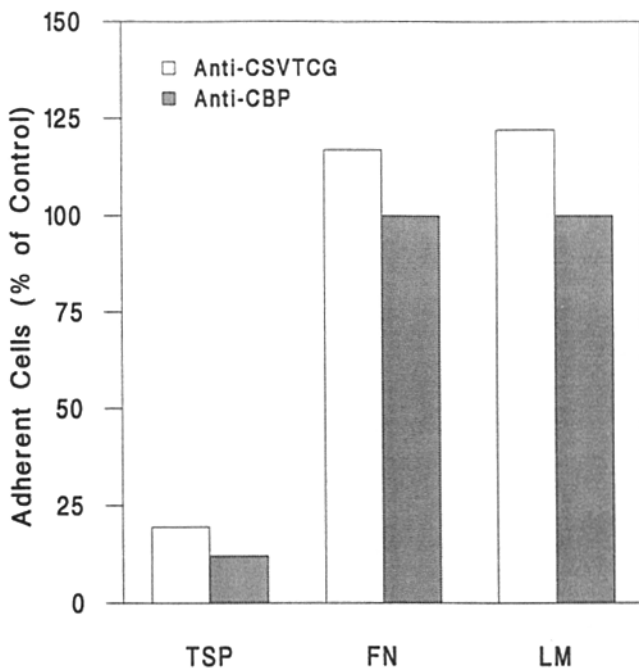

Figure 12. The effect of anti-CSVTCG binding protein antibody and anti-CSVTCG peptide antibody on TSP-dependent A549 tumor cell adhesion. A549 tumor cells (1 ml of a 4 × 10⁵/ml cell suspension in DME) were treated with either 200 μg/ml control rabbit IgG or 200 μg/ml anti-CSVTCG binding protein IgG for 30 min at 37°C. Excess antibody was removed by centrifugation and cells were resuspended in DME. Cells were then added to protein-coated microtiter dishes and the number of adherent cells determined after 1 h as previously described (31, 34). In the case of anti-CSVTCG antibody, TSP-coated dishes were incubated with either 100 μl of 100 μg/ml solution of anti-CSVTCG antibody or control goat IgG for 30 min at 37°C before addition of cells. The number of adherent cells were measured after 1 h. Approximately 200-400 cells adhered per 1 mm² of tissue culture plate coated with either TSP, fibronectin (FN) or laminin (LM) in the presence of control IgG (designated as control). All values were the average of duplicates which varied by <5%.

adhesion of tumor cells to the vessel wall because there were initially more tumor cells trapped in the lungs of the TSP-treated animals than in controls. In addition, the metastatic colonies in the TSP-treated animals were significantly larger than in the non-TSP treated animals suggesting that TSP may have had an effect on the growth and aggregate size of the cells. To determine whether high levels of circulating TSP could be playing a role in the pathogenesis of human cancer, plasma TSP levels of various cancer patients were measured. We found that patients with breast cancer, lung cancer, and colon cancer had circulating plasma TSP-levels two to threefold higher than normal controls or patients with noncancer illnesses (35). Similar results were found for patients with myeloproliferative disorders (17), and gynecologic malignancies (18). TSP could not only promote the adhesion of these human tumors and thereby promote metastasis formation but also enhance the metastatic potential of these cells. The latter conclusion was supported by the study of Castle et al. (7) who showed that production of TSP by human squamous carcinoma tumors correlated with the expression of the malignant phenotype because the antisense reduction of TSP message resulted in tumors that were nonmetastatic, slow growing, and highly differentiated. Therefore, tumor cells may utilize both endogenously expressed TSP and platelet-released TSP as anchors for attachment to the vascular basement membrane and as promoters of the malignant phenotype.

It is clear from a number of studies that TSP can bind to multiple cell surface receptors on the same cell or bind to different receptors on different cells. For example, platelets can bind TSP through GPII$_b$-III$_a$ (10, 30), the vitronectin-receptor (14), and GPI$_a$-II$_a$ (30). Smooth muscle cells (16), endothelial cells (16), U937 monocyte-like cells (16), and melanoma cells can bind TSP (28) through a vitronectin-like receptor. Squamous cell carcinoma cells bind TSP through an $M_r = 80,000/105,000$ protein that is not an integrin or GPIV (40).

The involvement in TSP cell adhesive interactions of GPIV or CD36, originally described as a monocyte specific antigen (12), remains controversial. Originally, Asch and colleagues (4) demonstrated that TSP binding to activated platelets, melanoma cells, and fibrosarcoma cells could be inhibited by a monoclonal antibody to GPIV, OKM5 (12). These cells contained GPIV on their surfaces and isolated GPIV could be shown to bind TSP in vitro. However, Aiken et al. (1) could not block platelet surface TSP expression with anti-GPIV antibody and the analysis was further complicated by the fact that anti-GPIV caused platelet activation. Platelets deficient in GPIV bound TSP to the same extent as normal platelets suggesting that other platelet receptors were capable of binding TSP (11). Anti-GPIV alone could not block TSP-mediated cell adhesion but did so weakly in the presence of high levels of heparin suggesting that GPIV is not a primary mediator of adhesion in this system despite the fact that previous in vitro assays performed by different groups show that isolated GPIV could bind TSP (2, 6). Initial studies showing that GPIV mediated TSP-monocyte binding (24, 25) have recently been called into question because the same results could be obtained by measuring the binding of TSP to a calcium precipitate formed by the addition of CaCl₂ to the binding medium used in the initial studies (26). Finally, two opposite results were obtained from GPIV transfection studies. Oquendo et al. (19) found that COS cells

transfected with GPIV showed no difference in TSP binding compared to controls. In contrast, Asch et al. (5) found that Jurkat cells transfected with GPIV bound TSP saturably whereas control cells showed little or no binding. One difference in these transfection studies is the cell type used. COS cells are adherent cells and Jurkat cells are suspension cells. It would be interesting to know if the GPIV-transfected Jurkat cells became adherent especially on TSP-coated surfaces.

As described above, TSP plays a major role in cellular adhesion. We recently identified a TSP cell adhesion domain that consisted of the hexapeptide sequence, CSVTCG (32). Synthetic CSVTCG peptides and homologous analogues promoted the adhesion of A549 lung carcinoma, B16-F10 melanoma cells, bovine aortic endothelial cells, and rabbit smooth muscle cells. These peptides inhibited platelet aggregation and the complex process of tumor cell metastasis which depends in part on TSP adhesive activity (27). We postulated that the way these peptides inhibited platelet aggregation and tumor cell metastasis was to bind to cell surface receptors specific for CSVTCG and TSP and thereby compete for endogenous TSP adhesive interactions.

To test this hypothesis, CSVTCG A549 tumor cell binding proteins were isolated and characterized in an in vitro TSP binding and cell adhesion assay. Chromatography of A549 cell detergent extracts on a CSVTCG peptide affinity column yielded one tightly-bound protein peak that was eluted at high pH. The protein appeared homogenous because it could be eluted as a single peak from an anion exchange column and analyzed by isoelectric focusing as a homogeneous band having a pI of 4.7. The protein appeared to be glycosylated because it bound to galactose, glucosamine, and mannose specific lectins. Consistent with the presence of glycoconjugate was the high 260 nm absorbance of the purified protein and the capacity of heparin to inhibit the binding interaction between TSP. On SDS gels under reducing conditions, the protein migrated as two major polypeptide bands spaced closely together with apparent molecular weights of 60,000 and 50,000 kD. Under nonreducing conditions, a single band of 50,000 kD was observed but in some preparations small amounts of dimers could be observed with molecular weights >100,000 kD. These results are consistent with the interpretation that the protein consists of two interchain disulfide-linked polypeptide chains that assume a more compact configuration when disulfide bonded.

The CSVTCG binding protein is surface exposed in A549 cells because it could be labeled with [^{125}I]iodine on intact cells. In addition, a rabbit polyclonal antibody raised against the protein inhibited adhesion and spreading of A549 cells to TSP but not to fibronectin or laminin indicating that not only is the CSVTCG binding protein surface exposed but it is also functioning as a TSP adhesion receptor. Blockage of the ligand for the CSVTCG binding protein on TSP with anti-CSVTCG peptide antibody also inhibited TSP-promoted cell adhesion and spreading. These results suggest that cells utilize the CSVTCG-binding protein to adhere and spread on TSP-coated tissue culture plates.

To determine whether the CSVTCG could function as a receptor for TSP in vitro, labeled CSVTCG binding protein was evaluated for its capacity to bind TSP. We found that CSVTCG binding protein interacted with TSP saturably and in an ion-dependent manner. The calculated binding constant was 0.3 μM or 2.3 times more favorable than the bind-

ing interaction of TSP with GPI$_a$-II$_a$, a high affinity TSP binding protein in platelets (41). The binding interaction did not involve RGD sequences because no significant binding was observed on GRGDS or fibronectin. The observation that heparin inhibited the interaction between the CSVTCG binding protein and TSP suggests that sulfated glycoconjugates may be involved in the binding interaction. It is possible that CSVTCG and adjacent downstream sequences containing arginine could provide secondary conformation dependent heparin binding domains in TSP. In support of this conclusion is the observation that CSVTCR binds heparin (unpublished data). Therefore under in vitro conditions, the CSVTCG-binding protein could function as a binding protein for TSP and the binding interaction could be mediated by sulfated glycoconjugates associated with the purified CSVTCG binding protein.

To determine whether the CSVTCG binding protein functions to bind CSVTCG containing proteins in vivo, CSVTCG cell association studies were performed. We found that A549 cells bound CSVTCG in a dose-dependent manner. The binding was inhibited by anti-CSVTCG binding protein antibody and CSVTCG was cross-linked to cell-associated CSVTCG binding protein. These experiments further suggest that CSVTCG binding protein functions as a cell surface receptor for proteins containing the CSVTCG sequence.

Further analysis of the CSVTCG binding protein is currently underway. The protein does not cross-react with antibodies against integrins, GPIV, or the laminin receptor (data not shown). Attempts at partial sequence analysis have been unsuccessful due to blocked end groups. This protein may represent a new class of adhesion molecules that could function in the TSP-adhesive interactions operative during cell-matrix interactions and during the pathogenesis of tumor cell metastasis.

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