Biological Activities of Peptides and Peptide Analogues Derived from Common Sequences Present in Thrombospondin, Properdin, and Malarial Proteins

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Abstract. Thrombospondin (TSP), a major platelet-secreted protein, has recently been shown to have activity in tumor cell metastasis, cell adhesion, and platelet aggregation. The type 1 repeats of TSP contain two copies of CSVTCG and one copy of CSTSCG, per each of the three polypeptide chains of TSP and show homology with peptide sequences found in a number of other proteins including properdin, malarial circumsporozoite, and a blood-stage antigen of *Plasmodium falciparum*. To investigate whether these common sequences functioned as a cell adhesive domain in TSP, we assessed the effect of peptides corresponding to these sequences and an antibody raised against one of these sequences, CSTSCG, in three biological assays which depend, in part, on the cell adhesive activity of TSP. These assays were TSP-dependent cell adhesion, platelet aggregation, and tumor cell metastasis. We found that a number of peptides homologous to CSVTCG promoted the adhesion of a variety of cells including mouse B16-F10 melanoma cells, inhibited platelet aggregation and tumor cell metastasis, whereas control peptides had no effect. Anti-CSTSCG, which specifically recognized TSP, inhibited TSP-dependent cell adhesion, platelet aggregation, and tumor cell metastasis, whereas control IgG had no effect. These results suggest that CSVTCG and CSTSCG present in the type I repeats function in the adhesive interactions of TSP that mediate cell adhesion, platelet aggregation, and tumor cell metastasis. Peptides, based on the structure of these repeats, may find wide application in the treatment of thrombosis and in the prevention of cancer spread.

THROMBOSPONDIN (TSP)[1] is a 450,000-dalton glycoprotein secreted by platelets in response to such physiological activators as thrombin and collagen (9). TSP comprises 3% of the total platelet protein and 25% of the total platelet-secreted protein (26). Although the precise biological role of TSP has yet to be fully established, it is generally accepted that TSP plays a major role in cell adhesion and cell–cell interactions. For example, TSP was found to promote the cell–substratum adhesion of a variety of cells, including platelets, melanoma cells, muscle cells, endothelial cells, fibroblasts, and epithelial cells (24). In addition, cells with very specialized functions such as keratinocytes (27) and osteoblasts (16) attach to TSP. Finally, TSP promotes the irreversible aggregation of platelets (23).

Thrombospondin has been postulated to play a role in malarial infection induced by only one strain of malaria, *Plasmodium falciparum*, and tumor cell metastasis induced by small cell carcinoma and mouse sarcoma. During malarial infection, TSP promotes adhesion of parasitized red cells to endothelial cells (15) and during tumor cell metastasis TSP promotes adhesion of mouse sarcoma cells to the vascular bed (20) and expression of the malignant phenotype of small cell carcinoma (3). Furthermore, TSP has been shown to share sequence homologies with malarial circumsporozoite proteins (8), which function in the cellular adhesion of the sporozoite stage of the malarial parasite. Recently, Robson et al. (17) further noted that the most significant homology is based around the consensus sequence WSPCSVTCG, which is present in three homologous copies in TSP (referred to as the type 1 repeats; 11), six copies in properdin and one copy each in all of the malarial circumsporozoite proteins studied to date including the blood-stage antigen of *P. falciparum*. Based on the evidence described above, we postulated that the sequences contained in the type 1 repeats of TSP must be playing a very fundamental role in ligand–receptor interactions mediating cellular adhesion. Therefore to test this hypothesis, the cell adhesive activities of synthetic peptides corresponding to these sequences were evaluated for activity in TSP-dependent cell–substratum adhesion assays and in the biological processes of platelet aggregation and tumor cell metastasis, which in part depend on the adhesive activities of TSP. In the present study we demonstrate that these sequences mediate TSP-dependent adhesive inter-

1. *Abbreviations used in this paper*: KLH, keyhole limpet hemocyanin; PRP, platelet-rich plasma; TSP, thrombospondin.

actions and play a role in platelet aggregation and tumor cell metastasis. Our results confirm the recent studies of Rich et al. (14) and Prater et al. (13) who showed that sequences containing VTCG can function as cell recognition sites.

Materials and Methods

Materials

All reagents, unless specified otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies were purchased from ICN Flow (Irvine, CA).

Cell Adhesion Assay

The cell adhesion assay was performed as previously described (22). Briefly, 2 μg of each peptide or TSP in 50 μl Hepes-buffered saline, pH 7.35 was dried under nitrogen in the wells of microtiter plates. 100 μl of a suspension containing 2×10^5 cells/ml in Hepes-buffered saline, pH 7.35, containing 100 μM $MnCl_2$, and 5 mM glucose was incubated in the peptide-coated well for 30–60 min, non-adherent cells removed by aspiration, wells washed and total adherent cells determined by protein assay. Cells adhering to BSA-coated wells were 2–5% of TSP-adherent cells and considered background.

Direct Enzyme-linked Immunoadsorbent Assay

ELISA assays were performed as previously described (25). Briefly, microtiter plates were coated with 2 μg of TSP or 2 μg peptide 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.

Peptide Antibody

CSTSCG 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., Rockford, IL). 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 five weeks after the first injection. Goat immunization and collection of serum was accomplished commercially through Hazelton Research Products, Inc. (Denver, PA).

Polyclonal anti-CSTSCG was affinity purified on Sepharose-KLH, followed by Sepharose-TSP. Briefly, IgG prepared by ammonium sulfate precipitation of 100 ml of anti-sera was dissolved in 50 ml of Hepes-buffered saline, pH 7.35 (column buffer), and passed over a 5 ml column of Sepharose containing 15 mg of coupled KLH which was previously equilibrated with column buffer. The flow-through fraction was 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. 1-ml fractions were collected in tubes containing 50 μl of 1 M Tris base to neutralize the final solution. Protein fractions were pooled, concentrated, and assessed for anti-TSP activity by ELISA and Western immunoblot analysis.

Platelet Aggregation

Platelet aggregation was performed as previously described (23). Briefly, blood obtained from healthy volunteers was centrifuged to obtain platelet-rich plasma (PRP). The stirred PRP was aggregated with ADP in the presence and absence of peptide and the extent of aggregation monitored with a Chrono-log Aggregometer by measuring the increase in light transmission as a function of time. Collagen-induced platelet aggregation was performed on washed human platelets isolated as previously described (21). Briefly, platelets preincubated for 1 min with peptide were stirred with collagen at a final concentration of 5 μg/ml and the aggregation monitored optically with an aggregometer.

Table I. CSVTCG and Related Sequences Found in Different Proteins and Encoded by cDNA

Protein	Sequence	Reference
TSP	CSTSCG, CSVTCG	11
Circumsporozoite protein	CSVTCG	4
Trap	CSVTCG	17
Properdin	CSVTCG	7
Glycoprotein 13 equine Herpes virus	CSVTCG	1*
Cytomegalovirus HHLF5 protein	CSVTCG	29*
Gene 53 protein Varicella-zoster virus	CSVTCG	6*
Rat liver apolipoprotein A-I	CSVGCG	12*
Nitrate reductase	CSVTCK	2

The single-letter abbreviations for the amino acid residues are *A*, Ala; *C*, Cys; *D*, Asp; *E*, glu; *F*, Phe; *G*, Gly; *H*, His; *I*, Ile; *K*, Lys; *L*, Leu; *M*, Met; *N*, Asn; *P*, Pro; *Q*, Gln; *R*, Arg; *S*, Ser; *T*, Thr; *V*, Val; *W*, Trp; and *Y*, Tyr. * Encoded by cDNA and predicted to be in one of the six possible regions of the respective gene.

Synthetic Peptides

Synthetic peptides were purchased from Multiple Peptide Systems, (San Diego, CA). The peptides were >95% pure as assessed by HPLC and stored under argon at −20°C to prevent oxidation. Solutions were prepared immediately before use. For cell adhesion experiments, 2 μg of peptide was adsorbed on the surface of microtiter wells by drying under nitrogen 50 μl of a 40 μg/ml solution of peptide dissolved in Hepes-buffered saline, pH 7.35. In all other experiments, peptide stock solutions were 5–10 mg/ml in Hepes-buffered saline, pH 7.35. The free thiol content of the peptide solutions was routinely checked spectrophotometrically by titration with Ellman's reagent (Pierce Chemical Co., Rockford, IL). Peptides containing free thiols were >90% in the reduced form. Peptides used for metastasis assays were endotoxin free as assessed by enzyme-linked immunoassay and were found non-toxic to cells during 24 h of culture.

TSP Purification

TSP was purified from Ca^{+2} ionophore A23187-activated platelets as previously described (26).

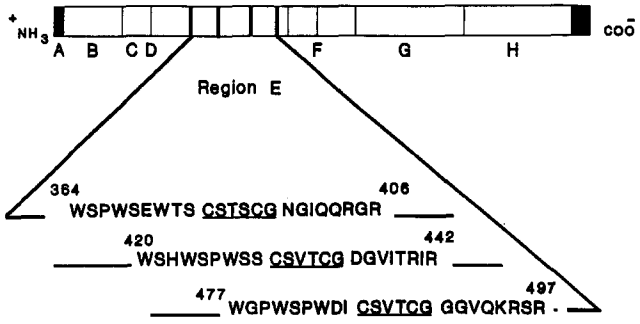

Figure 1. Representation of CSVTCG sequences in TSP. Sequence data obtained from reference 11.

Table II. Attachment of Various Cells to Microtiter Plates Coated with CSVTCG and Its Homologous Analogues

	Percent of TSP-adherent cells			
	Cell type			
Compound	B16-F10 mouse melanoma	A549 human lung adenocarcinoma	Bovine aortic endothelial cells	Rabbit smooth muscle
ANKHYF	8	10	0	17
VCTGSC	2	8	3	17
TCVCGS	10	10	6	8
CSVTCG	40	29	49	44
CSTSCG	37	23	39	22
Cyclic CSVTCG	30	29	25	43
C(Acm)SVTC(Acm)G	32	44	22	46
CSVTCR	92	90	99	159
CSTSCR	39	104	65	82
CRVTCG	92	79	80	82

Peptides CSVTCR and CSTSCR had the terminal carboxyl group in the amide form. Peptide C(Acm)SVTC(Acm)G contained acetaminomethyl blocked cysteine residues (28). The results are presented as percent of number of cells adherent to TSP-coated microtiter wells. Results of representative experiments are above. Percent TSP-adhesion was calculated as the number of cells adhering to the test compound divided by the number of cells adhering to TSP times 100. By definition adhesion to TSP is 100% and represents 500 adherent cells per mm^2. The number of adherent cells used to calculate % adhesion was the mean of triplicate determinations with standard deviations ranging from 1 to 5%.

Tumor Cell Metastasis Assay

Tumor cell metastasis was performed as previously described (20). Briefly, B16-F10 melanoma cells were cultured in DME containing 10% FCS. Cells were harvested in log phase with EDTA, washed, and suspended in Hepes-buffered saline. Each peptide was either coinjected with 1×10^5 B16-F10 mouse melanoma cells into a lateral tail vein of a C57BL/6 mouse or serially injected into separate lateral tail veins. In some experiments cells were injected first and then peptide. Animals were sacrificed 14 d later and lung tumor colonies were counted. Cell viability at the beginning and end of the metastasis experiments was >90% as measured by trypan blue exclusion.

Western Immunoblotting

Anti-CSTSCG antibody was characterized by Western immunoblotting using Pharmacia's Phast gel electrophoresis system. Approximately 100 ng of TSP 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 1% BSA in PBS-T and treated with 5 μg/ml of affinity purified goat anti-CSTSCG for 1 h, washed with PBS-T, developed according to the instructions provided with the VECTASTAIN ABC immunoperoxidase system, Vector Laboratories (Burlingame, CA).

Results

TSP-dependent Cell Adhesion

We chose to study CSVTCG and its related analogues because this sequence was present in two identical copies in the thrombospondin molecule. In addition, a computer search revealed that sequences analogous to this hexapeptide sequence were present or encoded by the cDNA of a number of apparently unrelated proteins suggesting some common function perhaps in ligand recognition (Table I). Furthermore, preliminary experiments showed that peptides derived from TSP sequences upstream of CSVTCG containing the sequence, WSP, had no activity in our adhesion assay (data not shown). In TSP, CSVTCG, and the related sequence CSTSCG occur in the type 1 repeats present in region E of the molecule which contains domains that interact with collagen and fibronectin (5), important adhesive extracellular matrix molecules (Fig. 1). Peptide CSVTCG and its analogues promoted

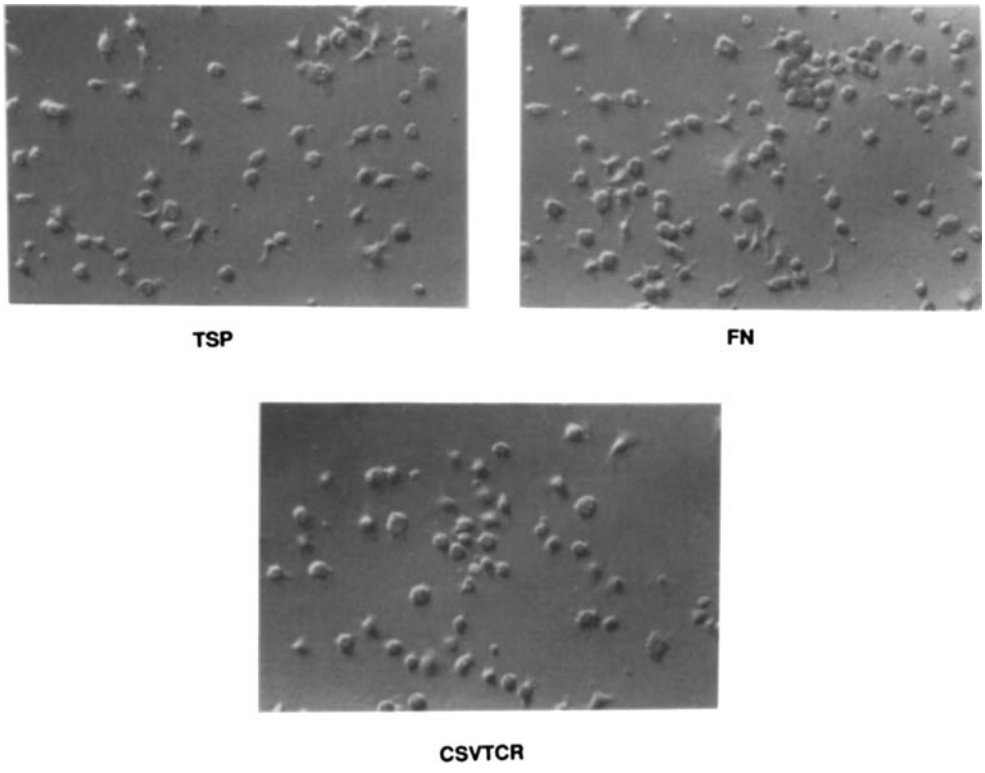

Figure 2. Adherent mouse B16-F10 mouse melanoma cells. Mouse B16-F10 melanoma cells were allowed to adhere for 30 min to microtiter plates coated with TSP, FN, and CSVTCR. Cells were photographed at a magnification of 300× using Hoffman interference microscopy.

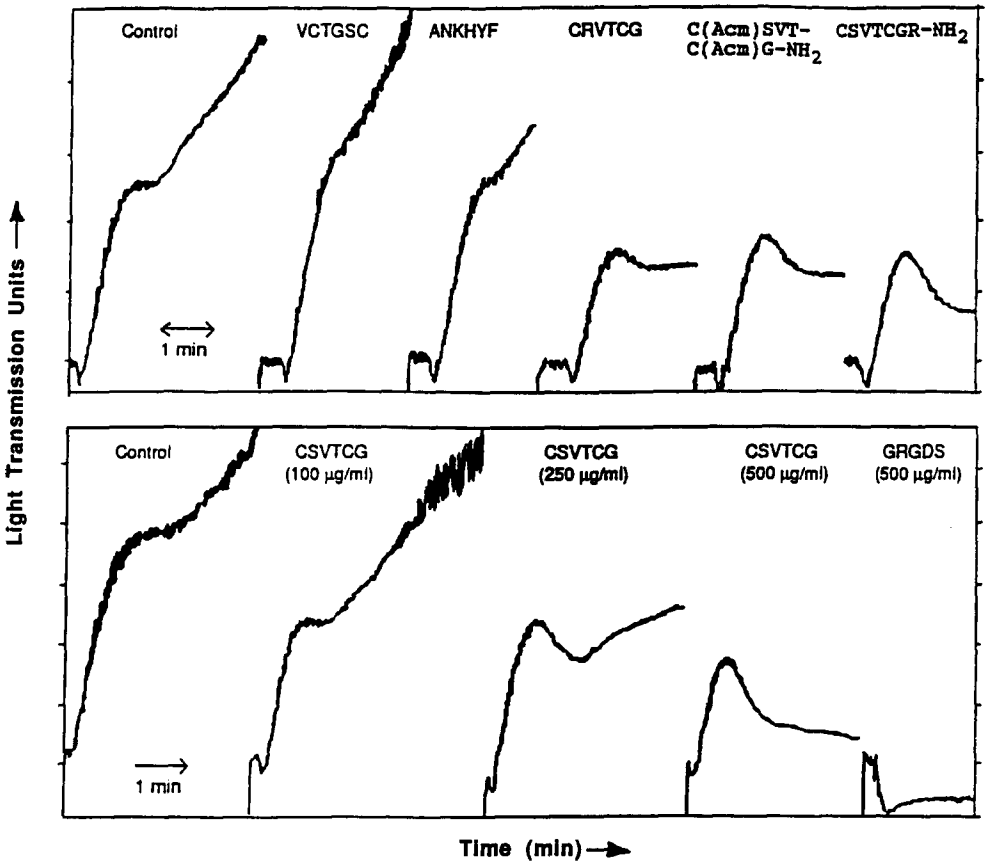

Figure 3. The effect of CSVTCG on ADP-induced platelet aggregation. Human platelet-rich plasma was aggregated with 4 μM ADP in the presence of various peptides. (*Top*) Platelets were aggregated in the presence of 500 μg/ml of peptide. (*Bottom*) Platelets were aggregated in the presence of increasing concentrations of CSVTCG shown in parentheses. For comparison, platelets were also aggregated in the presence of GRGDS, a peptide that totally inhibits aggregation. Aggregations were monitored on a Chrono-log aggregometer.

the attachment of a variety of cells including both normal and tumor cells (Table II). For example, B16-F10 mouse melanoma cells, A549 human lung adenocarcinoma cells, rabbit smooth muscle cells, and bovine aortic endothelial cells attached to the thrombospondin sequence CSVTCG 29–49% compared to TSP. In contrast, the control peptides, TCVCGS, VCTGSC, and ANKHYF, displayed little or no significant cell adhesive activity. In addition, derivatives of CSVTCG, cyclic CSVTCG, in which the two cysteine residues are joined by disulfide residues to form a five-membered ring structure, and C(Acm)SVTC(Acm), in which C(Acm) is an acetamidomethyl-blocked cysteine residue, promoted attachment of the four cell lines approximately to the same extent as CSVTCG. The substitution of R for G to yield CSVTCR displayed the greatest cell attachment activity of 90–159% as compared to TSP. The peptide CSTSCR obtained by substituting R for G in CSTSCG, a sequence present in the type 1 repeats of TSP, also displayed a high adhesive activity of 39–104% as compared to TSP. The human cancer cell line A549 adhered to this peptide to the same extent as TSP. In addition, the morphological appearance of B16-F10 melanoma cells on CSVTCR resembled that observed on fibronectin, displaying a flattened morphology with numerous pseudopodia (Fig. 2). Similar morphological changes on CSVTCR were observed for the other cells listed in Table II. It is possible that substitution of arginine for glycine or serine could increase the capacity of these peptides to bind cell associated glycosaminoglycans and thereby increase their adhesive activity. In support of this hypothesis is the observation that CRVTCG possessed heparin binding activity whereas CSVTCG did not (data not shown).

Platelet Aggregation

Peptide CSVTCG and its analogues inhibited ADP-induced platelet aggregation (Fig. 3). In contrast, the control peptides, ANKHYF and VCTGSC, displayed no activity. The active peptides specifically inhibited the formation of the ADP-induced second wave of platelet aggregation as indicated by the decreased extent of light transmission observed during the later stages of platelet aggregation shown in Fig. 3. The lower extent of light transmission observed at later times during aggregation in the presence of active peptides was due to the formation of smaller platelet clumps (Fig. 4). For example, Fig. 4 shows that in the absence of agonist no platelet clumps are formed, in the presence of agonist and buffer macroaggregates formed comprising more than 1,000 platelets per clump, while in the presence of agonist and CSVTCG only 10–50 platelets per clump were generated. The effect of peptides on aggregate size could not be attributed to an inhibition of platelet secretion since serotonin release was not affected (data not shown). These peptides also inhibited platelet aggregation induced by collagen. For example, Table III shows the percent inhibition observed for two representative peptides, CSTSCG and CSVTCG. At the highest dose tested, CSTSCG and CSVTCG inhibited aggregation by 96 and 100%, respectively. These results suggest that the peptide CSVTCG and its homologous analogues inhibit platelet aggregation by antagonizing those adhesive interactions that mediate platelet aggregation.

Tumor Cell Metastasis

Peptides CSVTCG, CSTSCG, and cyclic CSVTCG, when

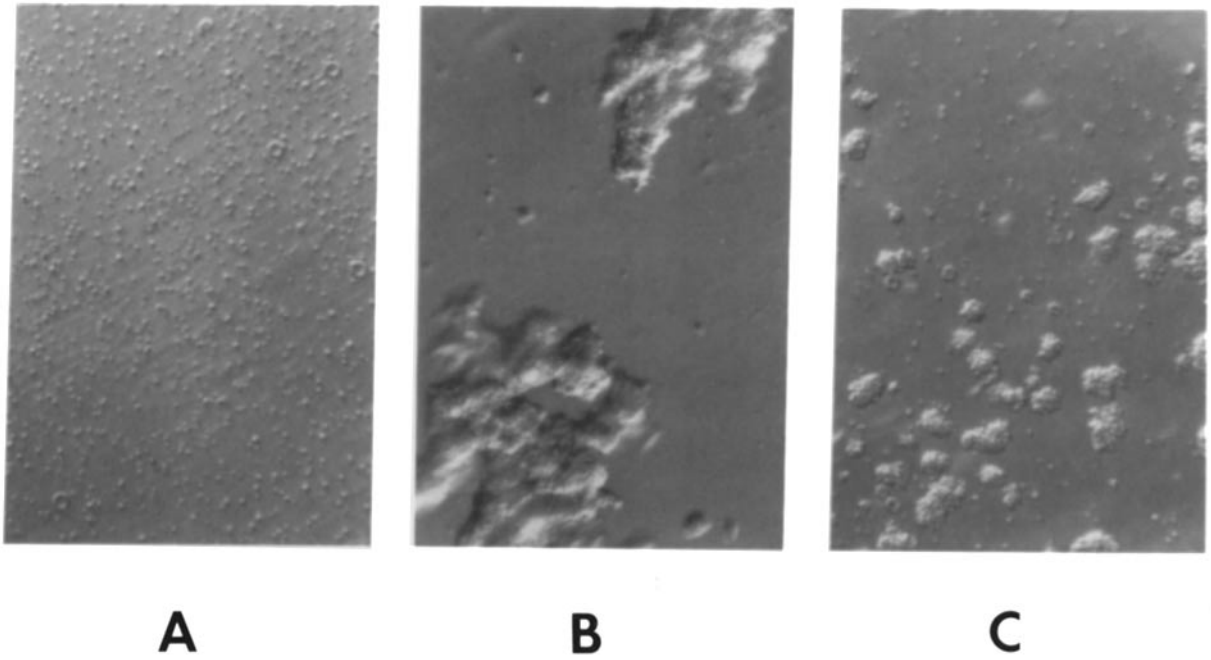

Figure 4. Aggregated platelets in the presence and absence of CSVTCG. Unaggregated platelets or platelets aggregated with 4 μM ADP for 5 min in the presence of buffer or 500 μg/ml CSVTCG were photographed at 300 times magnification using Hoffman interference microscopy. (*A*) Unaggregated platelets; (*B*) aggregated platelets in the absence of CSVTCG; (*C*) aggregated platelets in the presence of CSVTCG.

co-injected i.v. with 10^5 B16-F10 mouse melanoma cells, inhibited formation of lung tumor metastases by 42–85% in pathogen-free C57BL/6 mice (Table IV). Peptides had no effect on the viability of B16-F10 cells in vitro. Cell growth in culture was not inhibited by peptides at concentrations as high as 1 mg/ml after 24 h and cells were >90% viable as measured by trypan blue exclusion (data not shown). In addition, peptide solutions contained no endotoxin. To further rule out a direct effect of peptides on the metastatic potential of the cells, peptides were i.v. injected into one lateral tail vein followed by i.v. injection of tumor cells into the other lateral tail vein (Fig. 5) and the reverse i.e. tumor cells followed by peptide (Table IV). In each case, inhibition of tumor cell metastasis of 39–75% was observed. In contrast, the control peptides, ANKHYF and TCVCGS, had no significant effect on lung tumor colony formation, since the number of tumor colonies in the presence of these peptides was not statistically different from that observed in buffer control, $p < 0.01$. These results indicated that peptide CSVTCG and its homologous analogues have anti-tumor metastasis activity.

Table III. The Effect of CSVTCG and CSTSCG on Collagen-induced Platelet Aggregation

Compound	Concentration (μg/ml)	Percent inhibition
ANKHYF	500	0
CSVTCG	500	100
CSTSCG	500	96
CSVTCG	50	32
CSTSCG	50	26

Platelet aggregations were performed as described in Materials and Methods. Percent inhibition was calculated as the maximal increase in light transmission units of buffer control minus that in the presence of test compound times 100. The experiment shown represents a typical experiment obtained from a single normal blood donor. Qualitatively similar results were obtained from two other normal donors.

Table IV. Effect of Synthetic Peptides on B16-F10 Mouse Melanoma Cell Metastasis

Treatment	Number of lung tumors (mean ± SEM)	Percent inhibition
	Injection of cells followed by peptide	
Buffer	80 ± 10	0
TCVCGS	74 ± 24	8
Cyclic CSVTCG	35 ± 10	56
CSTSCG	49 ± 12	39
	Coinjection of cells and peptide	
Buffer	80 ± 12	0
CSVTCG	46 ± 5	42
CSTSCG	12 ± 2	85
Cyclic CSVTCG	28 ± 3	65

Each peptide (1 mg) was either coinjected with 1×10^5 B16-F10 mouse melanoma cells into a lateral tail vein of a C_{57} BL/6 mouse or serially injected into separate lateral tail veins, cells than peptide. Animals were sacrificed 14 d later and lung tumor colonies were counted. Each treatment group consisted of five animals. All metastasis experiments were repeated two to three times, showing qualitatively similar results. Representative experiments are shown.

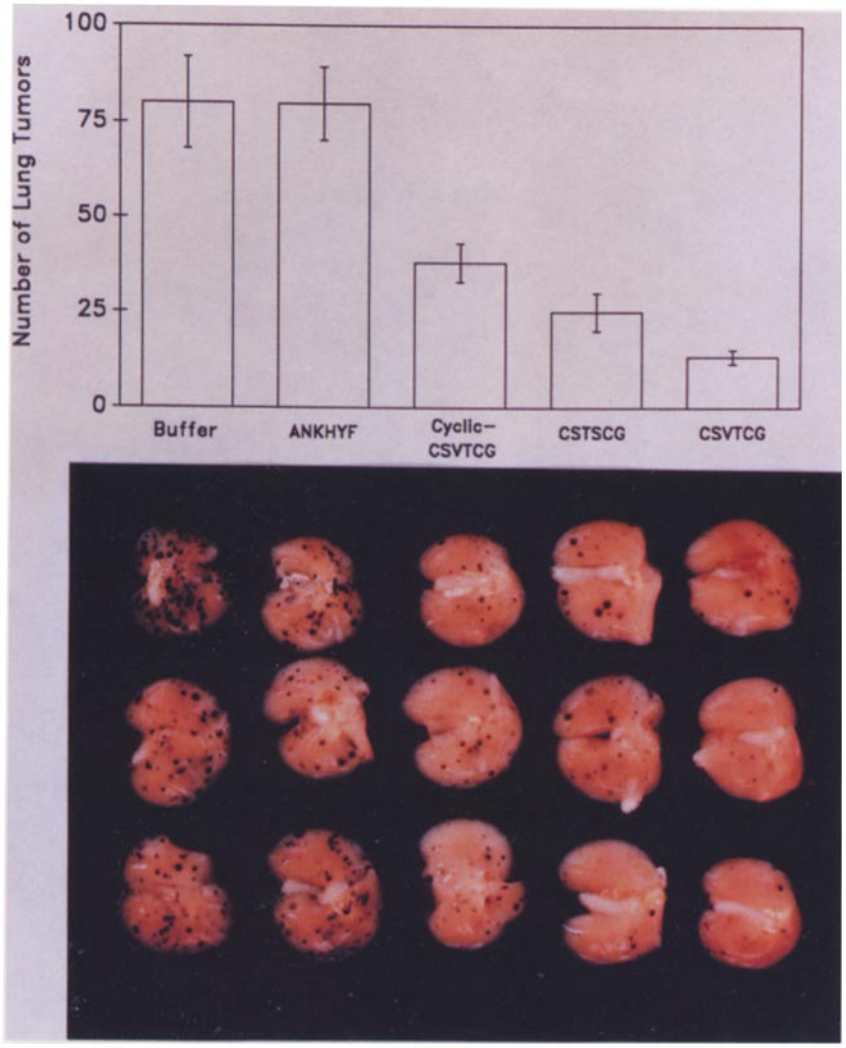

Figure 5. The effect of synthetic peptides on tumor cell metastasis. 1 mg of peptide was injected into one lateral tail vein of mice followed immediately by the injection of 1.0 $\times$ 10^5 B16-F10 mouse melanoma cells into the second lateral tail vein. Cells were harvested in EDTA and suspended in Hepes-buffered saline solution, pH 7.35, and peptides were dissolved in the same buffer. Mice were sacrificed after two weeks and the lung tumor colonies counted. Treatment groups contained five animals. The error bars represent the standard error of the mean. The photographs below the bar graphs show three lungs from each treatment group. The black spots are melanoma tumors. All metastasis experiments were repeated two to three times, showing qualitatively similar results. Representative experiments are shown.

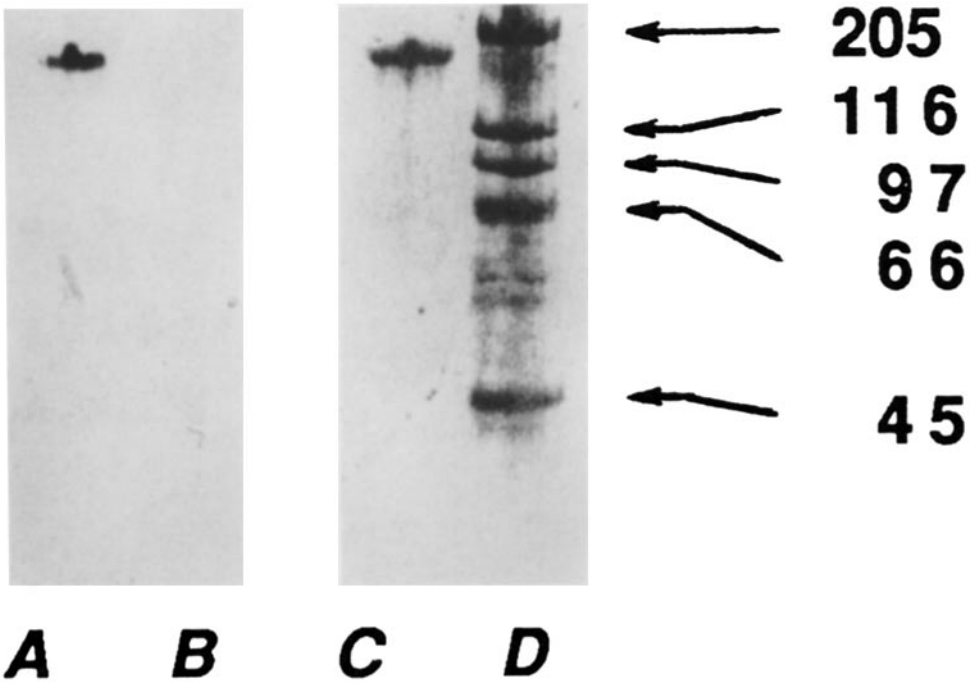

Figure 6. Analysis of anti-CSTSCG by western blotting. Purified TSP was separated under reducing conditions on an 8–25% polyacrylamide SDS-gel using the Pharmacia Phast System. Proteins were then electrophoretically transferred to nitrocellulose and either stained with silver or incubated with either 7.6 μg/ml solutions of affinity purified goat anti-CSTSCG or goat IgG in Tris-buffered saline containing 0.05% Tween 20. Bound antibody was detected with peroxidase-coupled rabbit anti-goat IgG. Lane *A*, TSP stained with antibody; lane *B*, TSP stained with control IgG; lane *C*, TSP silver stained; lane *D*, silver stained molecular weight standards.

Table V. Immunoreactivity of Goat Anti-CSTSCG Antibody

	Absorbance (405 nm)					
	BSA	Human TSP	Rat TSP	Mouse TSP	CSTSCG	CSVTCG
Goat IgG	0.037 ± 0.004	0.063 ± 0.002	0.042 ± 0.002	0.053 ± 0.004	0.048 ± 0.002	0.043 ± 0.002
Anti-CSTSCG	0.008 ± 0.006	0.713 ± 0.062	0.818 ± 0.039	0.169 ± 0.009	0.135 ± 0.030	0.186 ± 0.025

Immunoreactivity of anti-CSTSCG was measured by direct ELISA as described in Materials and Methods. Final antibody and IgG concentrations were 22 μg/ml. The average of three determinations is given ± the standard deviation. Rat and mouse TSP was purified by the same procedure used to purify human TSP.

The Oxidative State of Peptides

The oxidative state of the cysteine residues did not appear to influence the cell–substratum adhesive properties of the peptides examined in this study. For example, C(Acm)SVTC-(Acm)G, containing cysteine residues blocked with acetamidomethyl groups, which prevent inter- and intra-disulfide bond formation, and cyclic CSVTCG, which contains the two cysteine residues linked by disulfide bonds, both significantly promoted adhesion (Table II). Similarly, C(Acm)-SVTC(Acm)G inhibited platelet aggregation (Fig. 3) and cyclic CSVTCG prevented tumor cell metastasis (Fig. 5, Table IV). The sequence CSVTCG probably exists in TSP with the cysteine residues disulfide-linked, since the three free thiol groups present in TSP can appear anywhere in positions 687-1149 (19), while residues CSTSCG are contained in positions 373-378, and CSVTCG are contained in residues 429-434 and 486-491 of TSP.

Anti-peptide Antibody

To further establish that the type 1 repeats of TSP-promoted cell adhesive interactions, a polyclonal antibody to CSTSCG was prepared. The antibody was raised in a goat by immunizing the animal with peptide coupled to KLH. The final antibody was adsorbed with KLH and affinity purified on a TSP-Sepharose column. The affinity purified antibody recognized the 180,000 dalton chain of TSP (Fig. 6). In addition, by means of direct immunoadsorbent assay, the antibody reacted with CSTSCG, CSVTCG, human TSP, rat TSP, mouse TSP, but not with BSA (Table V). When adsorbed TSP was treated with this antibody and tested for cell adhesive activity, 70% of the cell adhesive activity of TSP was inhibited whereas the same antibody had no effect on fibronectin-mediated adhesion (Fig. 7). In addition, anti-KLH antibodies had no effect on TSP-mediated adhesion. Anti-CSTSCG at a concentration range of 33–70 μg/ml inhibited ADP-induced platelet aggregation and tumor cell metastasis by 73–80% (Fig. 8). These results further support the conclusion that CSTSCG is a cell adhesive domain in TSP and provide strong evidence that peptides homologous to the type 1 repeats of TSP function in cell adhesive mechanisms.

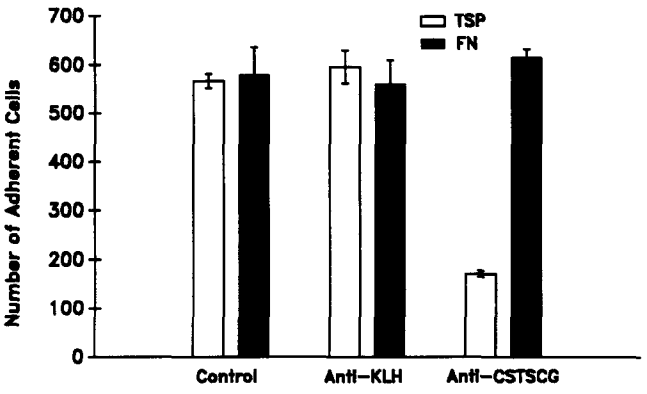

Figure 7. The effect of anti-CSTSCG on B16-F10 melanoma cell adhesion. Wells of an eight-well glass tissue culture slide were coated with TSP and fibronectin as previously described (24). Protein-coated surfaces were then treated with buffer or with 5 μl of a 500 μg/ml solution of either affinity purified anti-CSTSCG or anti-KLH for 30 min and cell adhesion performed as described (24). Solid bars represent adhesion to fibronectin and open bars represent adhesion to TSP. Treatment groups are designated below each set of bars. Error bars represent the standard deviation of triplicate samples. The experiment shown is a representative of two separate experiments that gave qualitatively similar results.

Discussion

Our data suggest that the conserved sequences, CSVTCG and CSTSCG, present in the type 1 repeats of TSP function as adhesive domains that mediate the cell interactions involved in platelet aggregation and tumor cell metastasis. During the course of this work Rich and co-workers (14) published a study demonstrating that 18–20 amino acid peptides containing the sequence VTCG found in the malarial circumsporozoite protein and in the protein from the sexual stage of *P. falciparum* promoted the attachment of a variety of human hematopoietic cell lines and two small cell carcinoma cell lines. Our studies are in agreement, since we find that peptides homologous to CSVTCG, which contain the VTCG sequence, promoted the attachment of a variety of cells including mouse melanoma cells and human lung adenocarcinoma cells. Shortly afterwards, Prater and co-workers (13) published a study showing that synthetic peptides corresponding to residues 424-42 and 481-99 of TSP promoted attachment of G361 cells but a peptide corresponding to residues 368-86 was inactive. The active peptides contained the sequence ASVTAG in place of CSVTCG and the inactive peptide contained the sequence ASTSAG in place of CSTSCG. The authors state that substitution of alanine for cysteine results in approximately a twofold loss in adhesive activity due to a lower coating efficiency of the alanine-containing peptide. Another difference between the work of Prater et al. (13) and the present study is that their mAb A4.1, the epitope for which localizes to the region of TSP containing the type 1 repeats, had no effect on platelet aggregation,

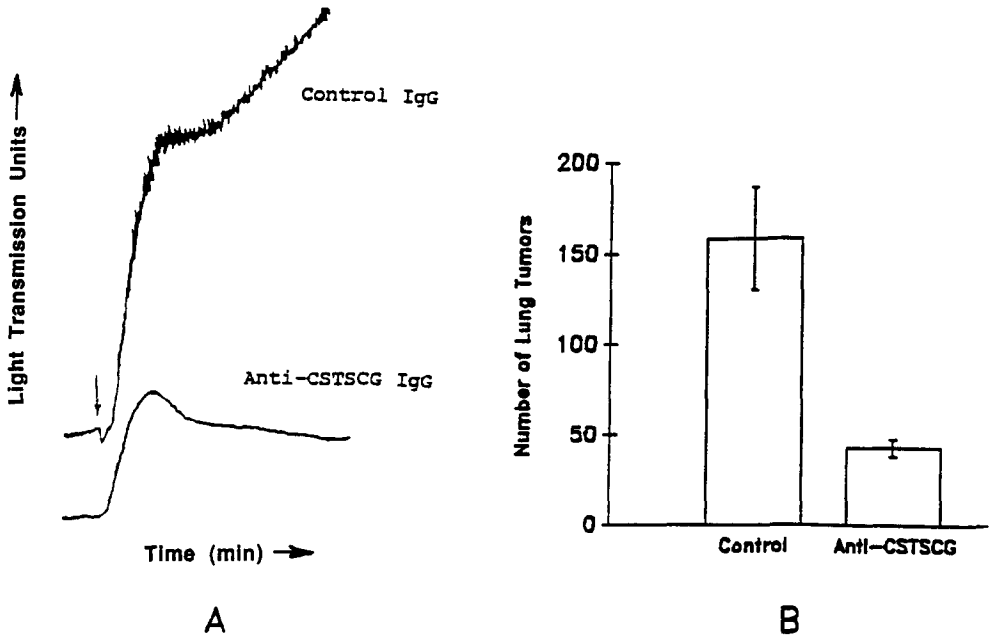

Figure 8. The effect of anti-CSTSCG on platelet aggregation and tumor cell metastasis. Panel A, human platelet-rich-plasma was aggregated in the presence of either 70 μg/ml of affinity purified anti-CSTSCG or 70 μg/ml non-immune control IgG. Panel B, 100 μg of either control IgG or 100 μg of affinity purified anti-CSTSCG was co-injected with 1.0×10^5 B16-F10 mouse melanoma cells into the lateral tail vein of mice (6 animals per group). Cells were harvested in EDTA and suspended in Hepes-buffered saline solution, pH 7.35, and antibodies dissolved in the same buffer. Antibodies had no effect on the viability of the cells as assessed by trypan blue exclusion. Mice were sacrificed after two weeks and the lung tumor colonies counted. The error bars represent the standard error of the mean. All metastasis experiments were repeated two to three times, showing qualitatively similar results. Representative experiments are shown.

whereas our anti-CSTSCG antibody inhibited platelet aggregation. A possible explanation for this difference is that A4.1 may not be directed against the same epitope as our antipeptide antibody. In our study, both CSTSCG and CSVTCG promoted cell adhesion suggesting that all three type 1 repeats per each of the three chains of TSP function as cell adhesive domains. The oxidative state of the cysteine groups did not appear to play a major role in the adhesion reaction since peptides having the cysteines blocked or oxidized promoted adhesion. For example, we found that peptide CSVTCG slowly oxidized in solution forming large intermolecular linked multimers that migrated with apparent molecular weights of 2,000 on SDS-gels. However, freshly prepared solutions were stable for at least 2 h.

Our work further suggests that these sequences may play an important role in the adhesive interactions mediated by TSP in the process of platelet aggregation and tumor cell metastasis. For example, we show that ADP-induced platelet aggregation is inhibited by CSVTCG and its homologous peptides. The platelet aggregates formed in the presence of these peptides are small and reversible suggesting that these compounds do not effect platelet fibrinogen binding but rather TSP platelet binding which has been shown to promote the formation of large irreversible aggregates (23). We think these peptides are inhibiting collagen-induced platelet aggregation by interfering with platelet collagen interaction. Further work is needed to clarify the mechanisms by which these peptides inhibit ADP and collagen-induced platelet aggregation.

We previously showed that TSP promoted the attachment of B16-F10 melanoma cells and that when intravenously injected with tumor cells in syngeneic mice, TSP promoted the arrest of B16-F10 cells in the lung with a subsequent increase in the formation of lung tumor colonies as compared to controls (20). In these early studies we postulated that endogenous TSP as well as exogenously added TSP promoted metastasis formation. The results of the present studies support the hypothesis that endogenous TSP mediates tumor cell metastasis in this model system. For example, the TSP-peptides, CSVTCG, and its homologous analogues, were found to inhibit metastasis by 39–85%. More inhibition was found when peptides were coinjected with cells or injected before injection of cells than when cells were injected followed by peptide. One interpretation of these results is that the peptides, structurally similar or identical to the adhesive domain of mouse TSP, antagonize the cell adhesive activity of endogenous TSP which, in part, mediates the arrest of tumor cells in the lung. We propose that endogenous TSP promotes tumor cell metastasis by providing circulating tumor cells with a provisional extracellular matrix from which they can escape the circulation and invade the surrounding tissue. This is accomplished by platelets which secrete large quantities of TSP and promote its incorporation into a fibrin matrix. The TSP-rich fibrin matrix traps tumor cells in the microvasculature and enables them to adhere, retract the endothelium, and invade the surrounding stroma. We feel this mechanism has great relevance to human cancer since patients with active metastatic disease have high levels of circulating TSP (18). In support of this interpretation for our model system are the recent studies of Lawler et al. (10) which indicate that the type 1 repeats in chicken, mouse, and human are highly conserved. For example, mouse TSP con-

tains two CSVTCG sequences and one CTVTCG sequence whereas human TSP contains two CSVTCG sequences and one CSTSCG sequence. Therefore, it is likely that CSVTCG and related peptides could antagonize the adhesive activity of endogenous mouse TSP.

To further establish that the sequences CSTSCG and CSVTCG functioned as TSP adhesive domains, a polyclonal antibody against CSTSCG was raised in a goat. The goat antibody was affinity purified on a TSP column, tested for reactivity against TSP by ELISA and Western immunoblot analysis and evaluated for activity in adhesion, aggregation, and metastasis assays. We found that anti-CSTSCG reacted with TSP, and blocked cell adhesion, platelet aggregation, and tumor cell metastasis. In the adhesion assay, even at the highest antibody doses tested, some cells still attached and spread on TSP suggesting that TSP must possess more than one cell adhesion domain. In platelet aggregation assays, the antibody inhibited only the second or secretion phase of platelet aggregation suggesting that platelet fibrinogen binding is not affected. Finally, the antibody significantly blocked tumor cell metastasis suggesting that the antibody interfered with the activity of endogenous mouse TSP. Since mouse TSP contains the related sequences, CTVTCG and CSVTCG (10), one might expect that anti-CSTSCG would bind mouse TSP and therefore block its activity. In fact, anti-CSTSCG antibody reacted with both mouse and rat TSP by direct ELISA supporting this proposed explanation for the antimetastatic activity of anti-CSTSCG.

In conclusion, these studies suggest that the conserved sequences homologous to CSVTCG in TSP and the malarial proteins may function in the binding of ligand receptor pairs that are fundamental in the cellular interactions governing malarial infection, hemostasis, and tumor cell metastasis. Elucidation of the receptors mediating these cellular events will provide more insight into the functional role of these conserved sequences.

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References

1. Allen, G. P., and L. D. Coogle. 1988. Characterization of an equine herpesvirus type 1 gene encoding a glycoprotein (gp13) with homology to herpes simplex virus glycoprotein c. *J. Virol.* 62:2850–2858.
2. Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy. 1989. Nitrate reductase of escherichia coli: completion of the nucleotide sequence of the nar operon and reassessment of the role of the alpha and beta subunits in iron binding and electron transfer. *Mol. Gen. Genet.* 218: 249–256.
3. Castle, V., J. Varani, S. Fligiel, E. V. Prochownik, and V. Dixit. 1991. Antisense-mediated reduction in thrombospondin reverses the malignant phenotype of a human squamous carcinoma. *J. Clin. Invest.* 87:1883–1888.
4. Dame, J. B., J. L. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Scheeinder, D. Roberts, G. S. Sanders, E. P. Reddy, C. L. Diggs, and L. H. Miller. 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite plasmodium falciparum. *Science (Wash. DC).* 225:593–599.
5. Dardik, R., and J. Lahav. 1987. The structure of endothelial cell thrombospondin. Characterization of the heparin-binding domains. *Eur. J. Biochem.* 168:347–355.
6. Davison, A. J., and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* 67:1759–1816.
7. Goundis, D., and K. B. Reid. 1988. Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature (Lond.).* 335: 82–85.
8. Kobayashi, S., F. Eden McCutchan, P. Framson, and P. Bornstein. 1986. Partial amino acid sequence of human thrombospondin as determined by analysis of cDNA clones: homology to malarial circumsporozoite proteins. *Biochemistry.* 25:8418–8425.
9. Lawler, J. 1986. The structural and functional properties of thrombospondin. *Blood.* 67:1197–1209.
10. Lawler, J., M. Duquette, and P. Ferro. 1991. Cloning and sequencing of chicken thrombospondin. *J. Biol. Chem.* 266:8039–8043.
11. Lawler, J., and R. O. Hynes. 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* 103:1635–1648.
12. Poncin, J. E., J. A. Martial, and J. E. Gielen. 1984. Cloning and structure analysis of the rat apolipoprotein A-1 cDNA. *Eur. J. Biochem.* 140: 493–498.
13. Prater, C. A., J. Plotkin, D. Jaye, and W. A. Frazier. 1991. The properdin-like type I repeats of human thrombospondin contain a cell attachment site. *J. Cell Biol.* 112:1031–1040.
14. Rich, K. A., F. W. George IV, J. L. Law, and W. J. Martin. 1990. Cell-adhesive motif in region II of malarial circumsporozoite protein. *Science (Wash. DC).* 249:1574–1577.
15. Roberts, D. D., J. A. Sherwood, S. L. Spitalnik, L. J. Panton, R. J. Howard, V. M. Dixit, W. A. Frazier, L. H. Miller, and V. Ginsburg. 1985. Thrombospondin binds falciparum malaria parasitized erythrocytes and may mediate cytoadherence. *Nature (Lond.).* 318:64–66.
16. Robey, P. G., M. F. Young, L. W. Fisher, and T. D. McClain. 1989. Thrombospondin is an osteoblast-derived component of mineralized extracellular matrix. *J. Cell Biol.* 108:719–727.
17. Robson, K. J. H., J. R. S. Hall, M. W. Jennings, T. J. R. Harris, K. Marsh, C. I. Newbold, V. E. Tate, and D. J. Weatherall. 1988. A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. *Nature (Lond.).* 335:79–82.
18. Smith, M., H. Switalska, V. Rothmman, D. M. Capuzzi, J. Katz, J. Treat, E. Besa, R. Joseph, and G. P. Tuszynski. 1990. Thrombospondin (TSP) levels in patients with malignancy. *Proc. Amer. Soc. Clin. Oncol.* 9:6.
19. Speziale, M. V., and T. C. Detwiler. 1990. Free thiols of platelet thrombospondin. Evidence for disulfide isomerization. *J. Biol. Chem.* 265: 17859–17867.
20. Tuszynski, G. P., T. B. Gasic, V. L. Rothman, K. A. Knudsen, and G. J. Gasic. 1987. Thrombospondin, a potentiator of tumor cell metastasis. *Cancer Res.* 47:4130–4133.
21. Tuszynski, G. P., and M. A. Kowalska. 1991. Thrombospondin-induced adhesion of human platelets. *J. Clin. Invest.* 87:1387–1394.
22. Tuszynski, G. P., and A. Murphy. 1990. Spectrophotometric quantitation of anchorage-dependent cell numbers using the bicinchoninic acid protein assay reagent. *Anal. Biochem.* 184:189–191.
23. Tuszynski, G. P., V. L. Rothman, A. Murphy, K. Siegler, and K. A. Knudsen. 1988. Thrombospondin promotes platelet aggregation. *Blood.* 72: 109–115.
24. Tuszynski, G. P., V. L. Rothman, A. Murphy, K. Siegler, L. Smith, S. Smith, J. Karczewski, and K. A. Knudsen. 1987. Thrombospondin promotes cell-substratum adhesion. *Science (Wash. DC).* 236:1570–1573.
25. Tuszynski, G. P., H. I. Switalska, and K. Knudsen. 1987. Modern Methods in Pharmacology. In Methods of Studying Platelet-Secreted Proteins and the Platelet Cytoskeleton, Vol. 4. Alan R. Liss, Inc., New York. 267–286.
26. Tuszynski, G. P., S. Srivastava, H. I. Switalska, J. C. Holt, C. S. Cierniewski, and S. Niewiarowski. 1985. The interaction of human platelet thrombospondin with fibrinogen. Thrombospondin purification and specificity of interaction. *J. Biol. Chem.* 260:12240–12245.
27. Varani, J., B. J. Nickoloff, B. L. Riser, R. S. Mitra, K. O'Rourke, and V. M. Dixit. 1988. Thrombospondin-induced adhesion of human keratinocytes. *J. Clin. Invest.* 81:1537–1544.
28. Veber, D. F., J. D. Milkowski, S. L. Varga, R. G. Denkewalter, and R. Hirschmann. 1972. Acetamidomethyl. A novel thiol protecting group for cysteine. *J. Am. Chem. Soc.* 94:5456–5461.
29. Weston, K. and B. G. Barrell. 1986. Sequence of the short unique region, short repeats, and part of the long repeats of human cytomegalovirus. *J. Mol. Biol.* 192:177–208.