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Heparin- and sulfatide-binding peptides from the type I repeats of human thrombospondin promote melanoma cell adhesion

(tryptophan/laminin/properdin repeats)

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ABSTRACT **Peptides from the three type I repeats of human endothelial cell thrombospondin, containing the consensus sequence -Trp-Ser-Xaa-Trp-, bind to sulfated glycoconjugates including heparin and sulfatide. The peptides are potent inhibitors for the binding of thrombospondin, laminin, or apolipoprotein E to these ligands. The thrombospondin peptides that inhibit heparin binding, but not adjacent peptides from the thrombospondin sequence containing the previously identified adhesive motif Val-Thr-Cys-Gly, promote melanoma cell adhesion when immobilized on plastic. Melanoma cell adhesion to the immobilized peptides is inhibited by soluble recombinant heparin-binding fragment of thrombospondin. The peptides also inhibit heparin-dependent binding of thrombospondin or laminin to human melanoma cells. The active peptides lack any previously identified heparin-binding consensus sequences and most do not contain any basic amino acids. Studies with homologous peptides showed that the tryptophan residues are required for binding. Adjacent basic residues in the second type I repeat enhance binding to heparin but not to sulfatide. Thus the type I peptides of thrombospondin define a distinct class of heparin-binding peptides.**

Heparin binding sites occur on many proteins (for review, see refs. 1 and 2). In many cases, binding to heparin has been shown to require specific positively charged side chains of lysine, histidine, or arginine, which may interact with the negatively charged sulfate esters or carboxylic acid groups on heparin. Searches for sequence homology among heparin binding proteins have identified several consensus sequences containing clusters of basic amino acids that may mediate heparin binding (1, 2).

Thrombospondin (TSP) is an extracellular matrix glycoprotein (for review, see ref. 3) that binds to heparin (4), heparan sulfate proteoglycans (5–7), and some sulfated glycolipids (6–8). This binding specificity plays a major role in some of the biological activities of TSP, including tumor cell spreading (9), chemotaxis (10), and binding and internalization of TSP by fibroblasts (11) and endothelial cells (12). Binding to heparin, sulfatide, and heparan sulfate proteoglycans is mediated by the amino-terminal globular domain of TSP (6, 7). The isolated amino-terminal domain, as a proteolytic fragment released by several proteases or as a recombinant fragment expressed in bacteria (13), exhibits heparin binding activity. A monoclonal antibody to this domain of TSP, A2.5, also specifically inhibits heparin and sulfatide binding. The amino-terminal domain contains consensus sequences characteristic of heparin binding proteins (1, 2, 14). Because heparin also inhibits sulfatide binding (8) and the recombinant heparin-binding domain binds avidly to sulfatide (D.D.R. and T.V., unpublished data), both ligands probably bind to the same site.

The trimer of 140-kDa carboxyl-terminal fragments of TSP remaining after proteolytic removal of the amino-terminal heparin-binding domains fails to bind to heparin or sulfatide (6, 7). However, this fragment also contains potential heparin-binding consensus sequences in the three type I properdin repeats (14, 15). Other proteins containing homologous repeat sequences have been shown to bind to sulfatide (16). These repeats also contain the amino acid sequence VTCG that supports adhesion of several cell types (17). Prater *et al.* (18) reported that a trimeric 50- to 70-kDa proteolytic fragment of TSP containing the type I repeats, but lacking the amino-terminal heparin-binding domain, can mediate melanoma cell adhesion; this adhesion is partially inhibited by heparin and other sulfated polysaccharides that inhibit TSP binding to heparin or sulfatide. The type I repeats may, therefore, be a second site for binding to sulfated glycoconjugates. However, the data also suggested that a distinct binding specificity that may utilize the VTCG sequence interacted with a separate cell receptor to promote melanoma cell adhesion.

Using a series of synthetic peptides containing the type I repeats of TSP, we have now identified specific peptides that are potent inhibitors of TSP binding to heparin or sulfatide. These are unusual inhibitors of heparin binding in that they lack the known consensus sequences and, in most cases, lack basic amino acids. We report here characterization of these peptides and demonstrate that they strongly inhibit heparin or sulfatide binding to the amino-terminal domain of TSP, promote melanoma cell adhesion, and inhibit binding of TSP and laminin to cells.

MATERIALS AND METHODS

Materials. TSP was purified from thrombin-stimulated human platelets as described (8). Recombinant heparin-binding fragments of human TSP, residues 1–174 (18 kDa) and 1–242 (28 kDa), and recombinant human apolipoprotein E (19) were provided by Bio-Technology General, Rehovot, Israel. Murine laminin purified from the Engelbreth–Holm–Swarm tumor was provided by Lance Liotta (National Cancer Institute). Monoclonal antibodies to TSP were provided by William Frazier (Washington University, St. Louis). TSP, its fragments, apolipoprotein E, bovine serum albumin (BSA)-peptide conjugates, and laminin were iodinated using Iodo-Gen (Pierce) as described (8). Heparin-BSA conjugate was prepared essentially as described (20). Bovine brain sulfatide was obtained from Supelco, and dipalmitoylphosphatidylcholine and cholesterol were from Sigma.

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Abbreviations: BSA, bovine serum albumin; TSP, thrombospondin.

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Peptides were synthesized corresponding to sequences of human TSP as indicated in Table 1 using standard Merrifield solid-phase synthesis protocols and *t*-Boc (*t*-butoxycarbonyl) chemistry. Peptides were analyzed by reverse-phase HPLC. Peptide solutions were neutralized by addition of dilute NaOH and stored at −20°C. Peptides were coupled through their cysteine residues to BSA by using *N*-succimidyl 3-(2-pyridylthio)proprionate (21).

Adhesion Assays. Human melanoma cell line A2058 (22) was maintained in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum. Melanoma cells were harvested by incubation with phosphate-buffered saline (PBS) containing 2.5 mM EDTA for 20 min at 37°C. The cells were centrifuged and viability (routinely >99%) was assessed by trypan blue exclusion. The cells were resuspended in medium and allowed to recover in suspension for 1 h. Attachment and spreading on plastic coated with proteins or peptides was determined as described (9).

Sulfatide and Heparin Binding. Laminin, apolipoprotein E, and TSP binding to sulfatide or heparin were determined using a solid-phase assay as described (8, 23). Sulfatide (0.2 μg per well for TSP binding and 0.6 μg per well for laminin binding) was immobilized in a mixture of 50 ng of phosphatidylcholine and 30 ng of cholesterol on polyvinyl chloride microtiter plates. Heparin-BSA (0.2 μg per well) was adsorbed by incubation in 50 μl of Dulbecco's PBS for 2 h at 37°C. Inhibition was determined using peptides diluted in 30 μl of 50 mM Tris·HCl (pH 7.8) containing 150 mM NaCl, 1 mM $CaCl_2$, 0.025% NaN_3, and 1% BSA (buffer I) or buffer alone and 30 μl of ^{125}I-labeled laminin or TSP (0.2 μg/ml).

Inhibition of ^{125}I-Labeled TSP or Laminin Binding to Cells. A2058 melanoma cells were harvested as described above and suspended in Dulbecco's PBS containing BSA at 1 mg/ml. In a final volume of 0.2 ml, 2×10^5 cells were preincubated for 15 min with potential inhibitors. Labeled protein was added and the cells were incubated on a rotating table for 1 h at 20°C. The cell suspension was transferred to 0.4-ml polypropylene microcentrifuge tubes (PGC Scientific, Gaithersburg, MD), which were preincubated with 1% BSA. Oil (Nyosil-50, 0.2 ml) was added and centrifuged for 1 min at 10,000 rpm in a Beckman microcentrifuge B. The upper phase was removed and the oil layer was washed with 0.2 ml of buffer I and recentrifuged. The supernatant fluid was aspirated, the bottom of the tube was excised, and the bound radioactivity was measured.

RESULTS

Several peptides from the type I repeats were tested for inhibition of TSP binding to heparin and sulfatide (Fig. 1 and Table 1). The peptides chosen flanked the VTCG sequence identified as an adhesive motif (17) but in most cases lacked clusters of basic amino acids with the predicted heparin-binding consensus sequence. Surprisingly, the sequences amino-terminal to the VTCG sequence were most active. Dodecapeptides from all three repeats inhibited TSP binding to both heparin and sulfatide with IC_{50} values ranging from 6 to 50 μM. The peptide from the third repeat was the most active inhibitor of heparin binding followed by the second and first repeats. The order of inhibition was different for TSP binding to sulfatide, where the second repeat peptide was the most potent inhibitor (Table 1). Two peptides from the amino-terminal heparin-binding domain of TSP were tested that contain consensus sequences for heparin binding, residues 23–32 and 77–83 (Table 1). Only the former peptide inhibited TSP binding to heparin, with an IC_{50} value of 60 μM. These peptides, however, did not inhibit TSP binding to sulfatide.

Since the most active peptides contained few or no basic amino acids, the possibility existed that the peptides were

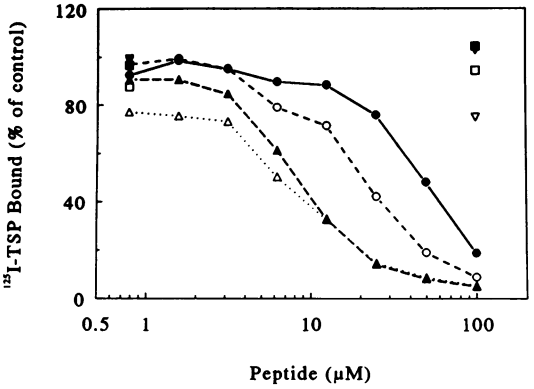

FIG. 1. Inhibition of ^{125}I-labeled TSP (^{125}I-TSP) binding to sulfated glycoconjugates by TSP peptides. Microtiter plate wells were coated with heparin-BSA and incubated with ^{125}I-labeled TSP at 0.2 μg/ml in the presence of increasing concentrations of the following peptides: ●, SPWSEWTSCSTS (184); ○, SHWSPWSSCSVT (185); ▲, GPWSPWDICSVT (186); △, CSVTCG (187); ■, VTCG (203); □, VTCGGGVQKR (204); ▼, VTCGDGVITR (205); ▽, TSCGNGIQQR (206). Binding is presented as a percent of control binding in the absence of inhibitor.

inhibiting by binding to a heparin-binding site on TSP rather than to the sulfated glycoconjugates. To examine this hypothesis, the peptides were tested as inhibitors of laminin and apolipoprotein E binding to heparin or sulfatide (Table 1). The same peptides were active as inhibitors of all three proteins and inhibited laminin binding to both substrates. Since it is highly unlikely that the same peptides could bind specifically to heparin-binding sites on three unrelated proteins, the activity of the peptides is due to binding to the sulfated glycoconjugates rather than to the protein. Lack of binding of the peptides to laminin or TSP was confirmed by the failure of labeled TSP or laminin to bind to the peptides immobilized directly on plastic or as BSA conjugates (results not shown).

Several peptides containing portions of the most active sequence from the second type I repeat were synthesized and tested as inhibitors of TSP and laminin binding to further define the active sequence for heparin binding (Table 1). VTCG was inactive, although a larger peptide containing this sequence, CSVTCG, was active. However, this peptide rapidly formed disulfide oligomers in solution, based on reversed-phase HPLC analysis. The inhibition curve was much more shallow than those for the other active peptides (Fig. 1), suggesting heterogeneous binding or that inhibition may be an artifact due to aggregation of the peptide. An analog of this peptide where the first cysteine was replaced with a serine, SSVTCG, was a weak inhibitor, except of TSP binding to sulfatide where it was 2-fold less active than CSVTCG. CSVT was also inactive, but addition of two residues on the amino terminus to give SSCSVT produced a weak inhibitor (IC_{50} = 85 μM) of TSP binding to heparin.

Several smaller peptides derived from the active sequence of the second type I repeat were also potent inhibitors (Table 1). A peptide containing the first eight residues, SHWSPWSS, was more active than the intact dodecapeptide in inhibiting TSP binding. A decapeptide (WSPWSSCSVT), lacking the first two amino acids of the dodecapeptide, was also a potent inhibitor. A peptide (WSPWSSCS) comprising the center eight residues, however, was much less active. By comparison with the sequences in the other two type I repeats, an optimal consensus sequence for binding may be defined: SXWSPWXS. The two tryptophan residues and the second serine residue were entirely conserved. To test the function of tryptophan in binding, a dodecapeptide was synthesized where the tryptophan residues were replaced

Table 1. Inhibition of ^{125}I-labeled TSP, laminin, or apolipoprotein E binding to heparin or sulfatide by synthetic peptides from TSP

Peptide	Repeat(s)	Sequence	IC_{50}, μM				
			TSP		Laminin		apoE
			Heparin	Sulfatide	Heparin	Sulfatide	Heparin
184	I	SPWSEWTSCSTS	47	14	36	42	4.2
186	III	GPWSPWDICSVT	8.5	27	15	35	52
185	II	SHWSPWSSCSVT	20	5.8	5	15	8
239	II	SHWSPWSS	5.2	10	11	12	250
244		SHASPASSCSVT	>200	>200	>200	>200	175
240	II	WSPWSSCS	60	100	100	≈100	150
241	II	WSPWSSCSVT	35	34	34	50	180
246		KRFKQDGGWSHWSPWSS	2.1	>100	3	>100	5.7
203	II and III	VTCG	>200	>200	>200	>200	
245	III	VTCGGGVQKRSRL	>200	>200	≈200	>200	
204	III	VTCGGGVQKR	>200	>200	>200	>200	
205	II	VTCGDGVITR	>200	>200	>200	>200	
187	II and III	CSVTCG	6	14	3	3.9	84
237		SSVTCG	58	30	100	85	
234	II and III	CSVT	>200	>200	>200	>200	
235		SSVT	>200	>200	>200	>200	
238	II	SSCSVT	85	>100	>200	>200	
206	I	TSCGNGIQQR	>200	>200	>200	>200	>400
P1	NT	RQMKKTR	>200	>200	>200	>200	
P2	NT	RKGSGRRLVK	60	>200	60	>200	

NT, amino terminal; apoE, apolipoprotein E.

with alanine, SHASPASSCSVT. This peptide was >100-fold less active than SHWSPWSS, the natural sequence in TSP. Thus, at least one of the two tryptophan residues is essential for activity.

The putative heparin-binding consensus sequence to the right of the VTCG sequence in the third repeat and a similar sequence to the left of the active sequence in the second repeat were also tested for activity (Table 1). A peptide containing VTCG and extending through the BBXB motif (VTCGGGVQKRSRL, where underlined residues indicate the basic motif) was inactive. Addition of the flanking BBXB motif to the second repeat (KRFKQDGGWSHWSPWSS), however, enhanced the inhibition of TSP or laminin binding to heparin ≈3-fold but markedly decreased the inhibition of both proteins binding to sulfatide.

To directly demonstrate binding of the active peptides to heparin, the peptide from the second repeat, peptide 246, was applied to a heparin affinity column (results not shown). The peptide was quantitatively bound when applied in 20 mM Tris buffer and eluted at 0.13–0.18 M NaCl in three experiments.

Peptides from the type I repeats significantly inhibited TSP and laminin binding to A2058 melanoma cells (Fig. 2). The order of activities for the peptides was the same as was observed for binding of the respective proteins to heparin. The peptide containing the extended second repeat (peptide 246) was most active, inhibiting TSP binding >90% at 10 μg/ml. At the concentrations used, dodecapeptides from all three type I repeats partially inhibited TSP and laminin binding to melanoma cells. Inhibition was dose-dependent (data not shown) and occurred at comparable concentrations to those needed to inhibit binding of the proteins to heparin. However, complete inhibition could not be demonstrated using the dodecapeptides because binding was nonspecifically enhanced by higher concentrations.

Heparin inhibited both TSP and laminin binding to the A2058 melanoma cells (Fig. 2). TSP binding was inhibited ≈90%, but ≈50% of laminin binding was resistant to inhibition by excess heparin. Laminin binding to A2058 melanoma cells was shown (23) to be partially heparin-dependent. Addition of peptide 246 to 1 μg/ml in the presence of heparin did not further inhibit binding of laminin (Fig. 3), indicating that inhibition by the peptide was due to competition for binding to a sulfated glycoconjugate rather than to a heparin-resistant protein receptor for laminin on the melanoma cells. For peptide at 10 μg/ml, the inhibition of laminin binding was partially reversed by addition of heparin, probably due to binding of the heparin to the peptide.

Several of the peptides when adsorbed onto plastic strongly promoted melanoma cell adhesion (Fig. 4). Activity in the adhesion assay was consistent with the ability of the peptides to inhibit TSP binding to heparin or sulfatide. Peptide 185 was more active than peptide 184 or 186 in both assays. The active subfragments of peptide 185 also promoted cell adhesion. None of the peptides containing VTCG promoted significant cell adhesion above background except CSVTCG. As was observed for inhibition of heparin binding, replacement of the first cysteine residue in CSVTCG with serine abolished activity in promoting melanoma cell adhe-

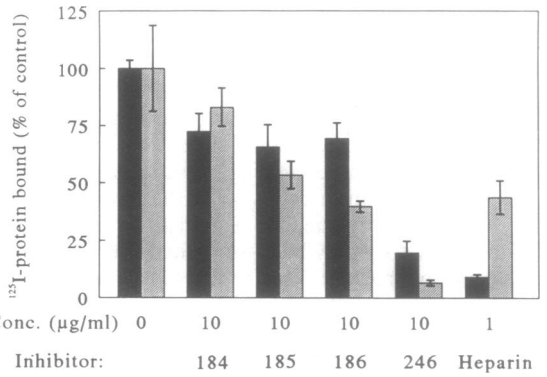

FIG. 2. Inhibition of ^{125}I-labeled laminin or ^{125}I-labeled TSP binding to A2058 melanoma cells by TSP peptides. Melanoma cells (2×10^5 cells in 0.2 ml) were incubated with ^{125}I-labeled laminin at 0.2 μg/ml (solid bars) or TSP at 0.2 μg/ml (hatched bars) alone or in the presence of peptides at 10 μg/ml from the first (peptide 184), second (peptides 185 and 246), or third (peptide 186) type I repeat of TSP or heparin at 1 μg/ml. The cells were centrifuged through oil to separate bound from free laminin or TSP and radioactivity in the cell pellet was quantified in a γ counter. The results are presented as a percent of control binding determined in the absence of peptide and are the mean ± SD of triplicate determinations.

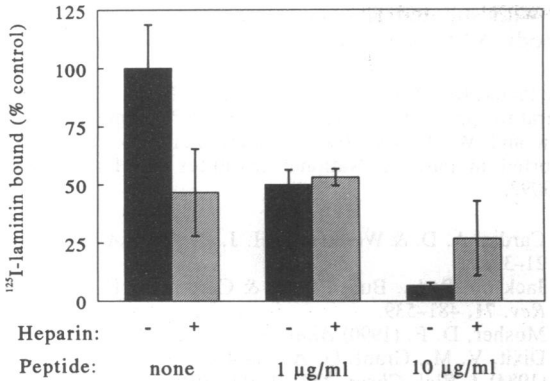

FIG. 3. Inhibition of ^{125}I-labeled laminin (^{125}I-laminin) binding to A2058 melanoma cells by heparin and/or TSP peptide 246 (KRFKQDGGWSHWSPWSS). Melanoma cells (2×10^5 cells in 0.2 ml) were incubated with ^{125}I-labeled laminin at 0.2 μg/ml alone (−, solid bars) or in the presence (+, shaded bars) of heparin at 0.1 μg/ml, peptide 246 at 1 or 10 μg/ml, or a combination of the two inhibitors. Results are presented as percent of control binding in the absence of inhibitor and are the mean ± SD of triplicate determinations.

sion. The two peptides from the amino-terminal domain of TSP did not promote melanoma cell adhesion.

Adhesion to the peptides was dose-dependent (Fig. 5). The extended peptide from the second repeat (246) was most active and, at 10 μg/ml, promoted extensive spreading of melanoma cells. Tryptophan residues were required for adhesion, as the dodecapeptide containing alanine replacements (peptide 244) was inactive. Lack of adhesion to peptide 244 was not due to inability of the peptide to bind to plastic, because iodinated peptide bound to polystyrene as efficiently as the active peptides (results not shown).

Apolipoprotein E and recombinant 18-kDa and 28-kDa heparin-binding fragments of TSP inhibited adhesion of A2058 melanoma cells to peptide 185 (Fig. 6). Inhibition was greater by apolipoprotein E than by either of the TSP fragments. This order of activity is consistent with the greater affinity of apolipoprotein E for heparin. Because both proteins bind to heparan sulfate, this result suggests that the peptides are promoting cell adhesion by binding to heparan sulfate proteoglycans on the melanoma cells.

DISCUSSION

We have defined a class of potent heparin-binding peptides from the type I repeat sequences of TSP that lack the currently known heparin-binding consensus sequences. Peptides from the three type I repeats bind to heparin and sulfatide and inhibit interactions of three heparin-binding proteins with sulfated glycoconjugates. Thus, these peptides may be general inhibitors of heparin-dependent adhesive proteins, growth factors, and coagulation enzymes. The peptides that inhibit binding to heparin strongly promote melanoma cell adhesion when immobilized on plastic. The peptides also inhibit some heparin-dependent interactions of laminin and TSP with human melanoma cells. The heparin-binding sequences from the type I repeats identified here are conserved in the second gene for TSP (24).

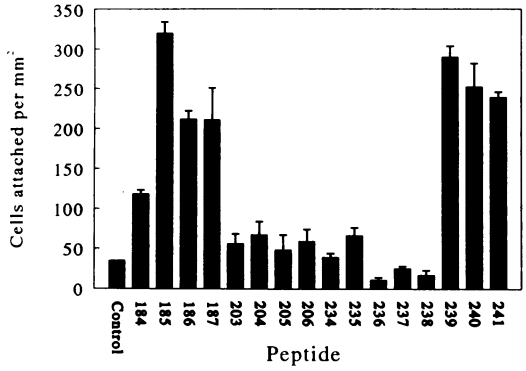

FIG. 4. Adhesion of A2058 melanoma cells to TSP peptides. Bacteriological polystyrene was coated with the indicated peptides at 200 μg/ml. A2058 melanoma cells (10^3 cells per mm^2) were added and incubated for 60 min at 37°C. Adhesion was determined microscopically and is presented as the mean ± SD (n = 6).

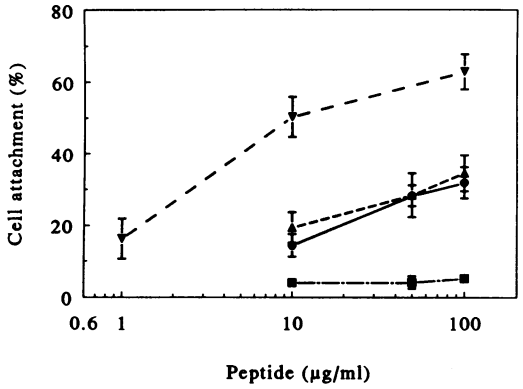

FIG. 5. Concentration dependence for A2058 melanoma cell adhesion to TSP peptides. Adhesion determined microscopically is presented as percent of cells applied (mean ± SD; n = 6) to plastic disks coated with the indicated concentrations of the following peptides: ●, SHWSPWSSCSVT (185); ▲, SHWSPWSS (239); ■, SHASPASSCSVT (244); ▼, KRFKQDGGWSHWSPWSS (246). Nonspecific adhesion was 1.9 ± 0.9%.

A minimal consensus sequence of -Trp-Ser-Xaa-Trp- was derived by comparison of the sequences of the most active peptides. At least one of the two tryptophan residues is essential for activity as replacement of both residues with alanine abolished activity for heparin or sulfatide binding and for promoting melanoma cell adhesion. This consensus sequence for heparin binding from TSP is conserved in several proteins containing properdin repeats (25), in most members of the cytokine receptor superfamily (26), and in some members of the transforming growth factor β superfamily (27).

The weak activity of SSCSVT and the enhanced activity of WSPWSSCSVT versus WSPWSSCS suggests that a second active sequence may be present. However, the tetrapeptides CSVT or VTCG are inactive. The peptide CSVTCG is active. Its activity may require disulfide-mediated polymerization, however, since replacement of the first cysteine with serine to prevent polymerization eliminates most activity. It remains to be determined whether two subsites are present or whether the differences in activity of the peptides are due to variation in conformation of a single active sequence among the peptides. VTCG is a potential adhesive sequence in the type I repeats for binding to protein receptors for TSP (17, 18). This sequence does not bind heparin, but the related peptide CSVTCG inhibits TSP binding to heparin. Because CSVTCG also inhibits binding of laminin and apolipoprotein E, however, this peptide is not useful as a specific probe of TSP binding to potential protein receptors recognizing the VTCG sequence.

In contrast to defined heparin-binding peptides containing clusters of basic amino acid residues (1, 2), tryptophan is a major determinant for heparin binding to the type I repeats. Tryptophan has also been implicated in heparin binding to antithrombin III based on chemical modification studies (28). Tryptophan residues were shown by crystallographic analysis to be directly involved in carbohydrate binding to an

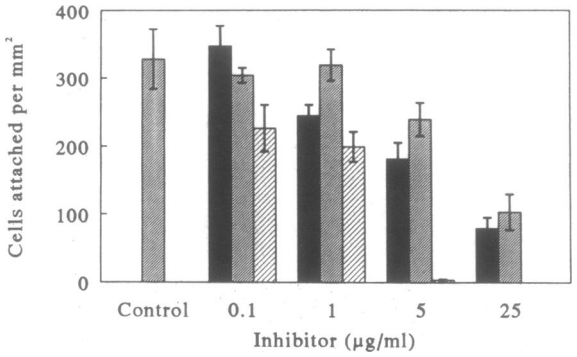

FIG. 6. Inhibition of melanoma cell adhesion to TSP peptides by heparin-binding proteins. Melanoma cells (1×10^3 cells per mm^2) in RPMI 1640 medium or medium containing the indicated concentrations of the 18-kDa TSP fragment (solid bars), the 28-kDa TSP fragment (shaded bars), or apolipoprotein E (hatched bars) were allowed to attach to polystyrene disks coated with peptide 185 (SHWSPWSSCSVT) at 200 μg/ml. Results are presented as mean ± SD ($n = 6$).

anti-carbohydrate monoclonal antibody through van der Waals interactions and hydrogen bonding (29). An analog of tryptophan, serotonin, was also reported to specifically bind to sialyl oligosaccharides (30). Further physical characterization of the interactions of the TSP peptides with heparin will be needed to establish the role of each tryptophan residue and the requirement for other amino acids including the conserved serine.

Although heparin competes for sulfatide binding to laminin and TSP, the type I repeat peptides reveal some differences between heparin and sulfatide binding activities. The heparin-binding consensus sequences from the amino-terminal domain of TSP weakly inhibit heparin binding. A similar sequence in the second type I repeat enhances inhibition of heparin binding when included with the tryptophan-containing heparin-binding sequence. Peptides containing the heparin-binding motif BBXB, however, failed in all cases to interact with sulfatide. Instead, addition of the basic sequence in the second repeat decreased sulfatide-binding activity. A heparin-binding motif in conjunction with VTCG, which was proposed to mediate sulfatide binding by proteins sharing type I repeat homologies (16), did not inhibit TSP binding to heparin or sulfatide and weakly inhibited laminin binding only to heparin. These findings are consistent with our previous report that heparin-binding consensus sequences in a denatured 50-kDa fragment of the A chain of laminin are sufficient for heparin but not for sulfatide binding (23).

TSP contains two potential heparin-binding sites. Both direct binding and antibody inhibition indicate that the amino-terminal domain is involved in some interactions with sulfated glycoconjugates on cells (3, 9, 10, 13). Based on the present results, the type I repeats contain strong heparin-binding sequences. Interaction of the 50- to 70-kDa fragment of TSP, which contains these sequences, with melanoma cells is partially heparin-dependent (18). However, the larger 140-kDa fragment containing the same sequences does not bind to heparin, sulfatide, or heparan sulfate (6, 7). Thus, the sequences are cryptic in this fragment. It cannot be established yet what conditions expose the sequence in the intact protein. To date, however, all reported interactions of intact TSP with sulfated glycoconjugates have been sensitive to antibody A2.5, which binds to the amino-terminal domain.

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