

Inhibition of Fibronectin Binding and Fibronectin-mediated Cell Adhesion to Collagen by a Peptide from the Second Type I Repeat of Thrombospondin

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Abstract. The platelet and extracellular matrix glycoprotein thrombospondin interacts with various types of cells as both a positive and negative modulator of cell adhesion, motility, and proliferation. These effects may be mediated by binding of thrombospondin to cell surface receptors or indirectly by binding to other extracellular matrix components. The role of peptide sequences from the type I repeats of thrombospondin in its interaction with fibronectin were investigated. Fibronectin bound specifically to the peptide Gly-Gly-Trp-Ser-His-Trp from the second type I repeat of thrombospondin but not to the corresponding peptides from the first or third repeats or flanking sequences from the second repeat. The two Trp residues and the

His residue were essential for binding, and the two Gly residues enhanced the affinity of binding. Binding of the peptide and intact thrombospondin to fibronectin were inhibited by the gelatin-binding domain of fibronectin. The peptide specifically inhibited binding of fibronectin to gelatin or type I collagen and inhibited fibronectin-mediated adhesion of breast carcinoma and melanoma cells to gelatin or type I collagen substrates but not direct adhesion of the cells to fibronectin, which was inhibited by the peptide Gly-Arg-Gly-Asp-Ser. Thus, the fibronectin-binding thrombospondin peptide Gly-Gly-Trp-Ser-His-Trp is a selective inhibitor of fibronectin-mediated interactions of cells with collagen in the extracellular matrix.

CELL-MATRIX interactions are important regulators of cellular differentiation during development and of several pathological processes including hemostasis, wound repair, tumorigenesis, and tumor metastasis. Thrombospondin is an extracellular matrix glycoprotein, derived from platelet α -granules and secreted by many cell types *in vitro*, that may play a role in each of these processes (reviewed in Frazier, 1991; Mosher, 1990). The biological responses of cells to thrombospondin are complex. Thrombospondin can either promote or inhibit adhesion (Roberts et al., 1987; Lahav, 1988), promote cell migration but inhibit migration of the same cells to other attractants (Taraboletti et al., 1987, 1990), and promote growth of smooth muscle cells (Majack et al., 1988) but inhibit growth of endothelial cells (Taraboletti et al., 1990). These opposing effects on cell behavior may be mediated directly by binding of thrombospondin to specific cell surface receptors (reviewed in Frazier, 1991), which are presumed to transduce information to the cell. Alternatively, thrombospondin may influence cell behavior indirectly by binding to other matrix components, including collagens, fibronectin, laminin, proteoglycans, and some proteases, thereby altering their biological properties.

Several of the ligand binding specificities of thrombospondin have been localized to a 70-kD protease-resistant core fragment of this protein, which contains the type I repeats of thrombospondin-1 (Lawler and Hynes, 1986). This domain mediates interaction of thrombospondin with fibronectin, laminin, collagens, and fibrinogen. This fragment or synthetic peptides, derived from its sequence or related sequences from the homologous malarial circumsporozoite protein, are adhesive for lymphoid and melanoma cells (Prater et al., 1991; Rich et al., 1990; Tuszynski et al., 1992). The peptides CSVTCG and CSTSCG from the thrombospondin type I repeats inhibit metastasis of melanoma cells in a murine lung colonization assay (Tuszynski et al., 1992). A 70-kD proteolytic fragment of endothelial thrombospondin that contains the type I repeats binds with high affinity to endothelial cells (Dardik and Lahav, 1991).

Two classes of biologically active peptides have been derived from the 70-kD core region of thrombospondin, containing the consensus sequences Val-Thr-Cys-Gly or Trp-Ser-Xaa-Trp-Ser. The former sequence promotes cell adhesion and may mediate interactions of thrombospondin with sulfated glycoconjugates and CD36 (Prater et al., 1991; Asch et al., 1992). The latter sequence binds specifically to sulfated glycoconjugates and promotes cell adhesion and chemotaxis (Guo et al., 1992a,b). The latter sequence is also conserved in members of the cytokine receptor (Bazan, 1990) and transforming growth factor β superfamilies (Wharton et al.,

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1991) and is essential for processing and function of at least two of the cytokine receptors (Miyazaki et al., 1991; Yoshimura et al., 1992).

Although most interactions of cells or proteins with peptides containing the consensus sequence Trp-Ser-Xaa-Trp are mediated by sulfated glycoconjugates (Guo et al., 1992c), when used as a control protein, human plasma fibronectin was found to bind unexpectedly avidly to a 17 amino acid peptide from the second type I repeat of thrombospondin, KRFKQDGGWSHWSPWSS. We have identified a hexapeptide within this sequence that accounts for this interaction and demonstrate that this peptide interacts primarily with the gelatin-binding domain of fibronectin. Furthermore, the peptide is a potent inhibitor of fibronectin binding to gelatin and of fibronectin-mediated cell adhesion to a gelatin or collagen matrix.

Materials and Methods

Materials

Human platelet thrombospondin was purified according to the previously described method (Roberts et al., 1985). Fibronectin was isolated from the platelet-depleted plasma as described (Akiyama and Yamada, 1985). Proteolytic fragments of fibronectin were obtained from Telios Pharmaceuticals, Inc. (San Diego, CA). Recombinant fragments of fibronectin were provided by BioTechnology General, Ltd. (Rehovot, Israel). Gelatin was from ICN Biomedicals, Inc. (Costa Mesa, CA), and type I collagen was from Collaborative Research, Inc. (Bedford, MA). Other control proteins were obtained from Sigma Chemical Co. (St. Louis, MO). Thrombospondin, goat anti-mouse IgG, and fibronectin were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) as previously described (Roberts et al., 1985).

Peptides were synthesized that corresponded to sequences of human thrombospondin-1 deduced from a cDNA sequence for *THBS1* (Lawler and Hynes, 1986). The peptides used in this study were synthesized on a peptide synthesizer (model 9600, Bioscience, San Rafael, CA) using standard Merrifield solid phase synthesis protocols and t-Boc chemistry. Sequences of the peptides used in these studies are presented in Tables I and II. Peptides were analyzed and, for some experiments, further purified by reverse-phase

HPLC using a C18 column. Three independent syntheses of peptide 300 (GGWSHW) were used in the experiments and had identical activities in inhibition assays but differed in their activities for direct binding when immobilized. Two were analyzed by californium desorption time of flight mass spectroscopy and gave the expected molecular ion at $m/z = 729.8$. The preparation with higher activity for direct binding gave a minor ion at $m/z = 785.9$, suggesting partial modification with *t*-butyl. Identities of some of the longer peptides, including peptide 246, were verified by complete amino acid sequence analysis. Peptide solutions were neutralized by addition of dilute NaOH and stored in solution at -20°C .

Ligand Binding Assays

Direct binding of fibronectin and other proteins to immobilized peptides were determined as previously described (Guo et al., 1992a,b). Briefly, the peptides were adsorbed on polyvinyl chloride microtiter wells. Saturation densities and adsorption capacities for many of the peptides used in these experiments have been previously reported (Guo et al., 1992b). Adsorption of the active peptide GGWSHW to plastic was determined after labeling by reductive methylation using NaB^3H_4 (Dupont-New England Nuclear, Boston, MA) to a specific activity of $0.69 \mu\text{Ci}/\mu\text{g}$. The peptide adsorbed to polyvinyl chloride microtiter plate wells with an adsorption constant of $10 \mu\text{M}$ and a capacity of 445 pmol/well .

The unbound peptides were removed by washing, and the wells were incubated for 0.5 h in Tris-BSA (50 mM Tris HCl, pH 7.8, 110 mM NaCl, 1% BSA (fatty acid and globulin-free, Sigma Chemical Co.), 0.1% NaN_3). The wells were washed and incubated with $30 \mu\text{l}$ of $0.4 \mu\text{g/ml}$ [^{125}I]fibronectin for 2 h at 25°C . The wells were washed three times with Dulbecco's PBS and cut from the plate, and the bound radioactivity was counted. For some experiments, unlabeled fibronectin or a gelatin-binding fragment was used in place of the labeled protein. Binding was detected using monoclonal antibody 191 (Newman et al., 1987), and [^{125}I]goat anti-rat IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Competitive inhibition of fibronectin binding to the immobilized peptides by soluble peptides or proteins was determined by including serial dilutions of the inhibitors to be tested and a final concentration of $0.2 \mu\text{g/ml}$ of [^{125}I]fibronectin.

Binding of [^{125}I]fibronectin to gelatin or type I collagen were determined using the ligands adsorbed on polyvinyl chloride microtiter plates. To determine binding of fibronectin to thrombospondin, the unlabeled protein ($4 \mu\text{g/well}$) was adsorbed on Immulon-2 Removawells by incubation for 3 h at 37°C . The wells were emptied and incubated with PBS-1% BSA buffer for 30 min. Binding of labeled fibronectin and inhibition were determined as described above except that the incubation was for 2 h at 37°C in PBS-BSA buffer.

Table I. Fibronectin Binding to Immobilized Peptides

Peptide/origin*	Sequence	FN Bound %‡
184 I ¹	SPWSEWTSCSTS	0.3
185 I ²	SHWSPWSSCSVT	0.2
186 I ³	GPWSPWDICSVT	0.3
245 I ³	VTCCGGVQKRSRL	0.2
246 I ²	KRFRKQDGGWSHWSPWSS	12.2
256 I ²	GGWSHWSPWSS	5.6
259 A	GGWSHASPWSS	0.4
263 I ²	KRFRKQDGGWSHWSP	5.7
266 A	KRFRKQDGGASHASP	0.1
292 I ²	WSHWSP	1.1
300 I ²	GGWSHW	51.0
322 I ²	QDGGWS	0.2
317 I ¹	DGWSPW	0.2
318 I ³	GGWGPW	0.3
319 A	GTWSEW	0.3
320 A	GFWSEW	0.3
297 C-term	THYRWRLSHRPKTGFI RV	0.2

* The synthetic peptides were derived from the first (I¹), second (I²), or third type I repeat of thrombospondin (I³) or from the carboxyl-terminal globular domain (C-term). Peptides labeled A are analogs of the thrombospondin sequences with amino acid substitutions as indicated.

‡ Fibronectin binding to the immobilized peptides was determined using microtiter plate wells coated with $100 \mu\text{g/ml}$ of each peptide.

Table II. Specificity for Inhibition of Fibronectin Binding to Immobilized Peptide GGWSHW

Peptide	Sequence	IC ₅₀ μM*
300	GGWSHW	29 ± 11
338	GGWTHW	150
339	GGWAHW	280
333	GGWSKW	>300
334	GGYSHW	>300
335	GGWSHY	>300
340	AAWSHW	>300
341	DGWSHW	>300
342	GWSHW	>300
322	QDGGWS	>300
292	WSHWSP	>300
246	KRFKQDGGWSHWSPWSS	≈200
256	GGWSHWSPWSS	>300
259	GGWSHASPWSS	>300

* Concentrations of peptide required for 50% inhibition of fibronectin binding to immobilized peptide GGWSHW.

For determination of the pH-dependence for inhibition of fibronectin binding to gelatin, a series of buffers of constant ionic strength, 0.15, were prepared (McKenzie, 1978) containing 1% BSA and used in place of the PBS-BSA buffer. Tris-HCl, Tris-citrate, sodium phosphate, and sodium acetate buffers were used.

Adhesion Assays

Human breast carcinoma cell line MDA-MB-435s (American Type Culture Collection, Rockville, MD) and human melanoma cell line A2058 (Todaro et al., 1980) were maintained in monolayer culture at 37°C with 5% CO₂ in Roswell Park Memorial Institute 1640 medium containing 10% FBS. Subconfluent cells were trypsinized and resuspended in RPMI 1640 medium containing 0.1% BSA. Gelatin or fibronectin were coated on plastic disks in 24 well plates and incubated for 2 h at 37°C. After the supernatant was removed, the disks were blocked with 1% BSA-Tris buffer, pH 7.8 for 30 min at room temperature. The disks were washed twice with PBS, and 0.4 ml of RPMI 1640 medium containing 0.1% BSA (and 2 μg/ml fibronectin for wells containing gelatin-coated disks) and the indicated concentrations of inhibitory peptides were added to the wells. A suspension of 10⁵ cells in 0.1 ml was added to the wells and incubated at 37°C with 5% CO₂ for 1 h. The disks were washed to remove nonadherent cells and stained. Adherent cells were counted microscopically.

Results

Several synthetic peptides containing portions of the type I repeat sequences of human thrombospondin-1 were tested for binding to fibronectin in a solid phase assay (Fig. 1 and Table I). Of the larger peptides tested from the three type I repeats or the carboxyl terminal domain of thrombospondin, only peptide 246 (KRFKQDGGWSHWSPWSS) bound labeled fibronectin. Binding did not require the amino terminal basic residues of peptide 246, based on the activity of the peptide 256 (GGWSHWSPWSS). At least two Trp residues were required, based on the activity of peptide 263 (KRFKQDGGWSHWSP) but not of peptide 266 (KRFKQDGGASHASP). The central Trp residue in peptide 256 is required for activity, since the peptide GGWSHASPWSS was inactive (Table I). Among the series of overlapping peptides QDGGWS, GGWSHW, WSHWSP, and SHWSPWSS, only GGWSHW was strongly active (Table I). At saturating concentrations of this peptide, 100 μg/ml, more than 50% of the added fibronectin was bound to the latter peptide (Fig. 1). The active peptide is from the second type I repeat of

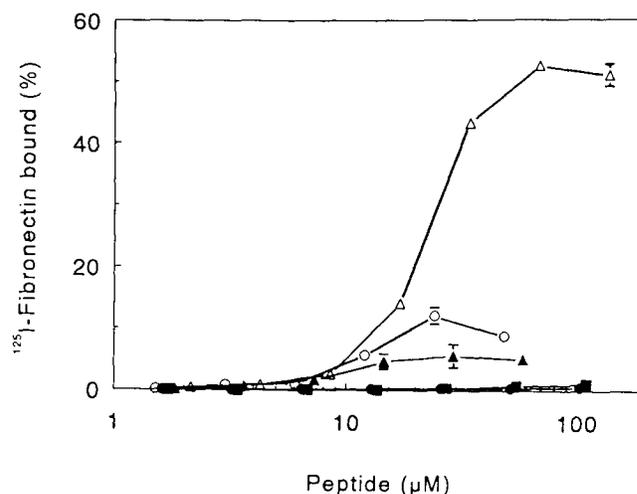


Figure 1. Binding of fibronectin to immobilized thrombospondin peptides. The indicated concentrations of the peptides SHWSPWSS (●), KRFKQDGGWSHWSPWSS (○), KRFKQDGGWSHWSP (▲), GGWSHW (Δ), or GGWSHWSP (■) were adsorbed on polyvinyl chloride microtiter plate wells by incubation for 3 h at 25°C. Binding of 0.5 μg/ml [¹²⁵I]fibronectin to the adsorbed peptides was determined at 25°C as described in Materials and Methods.

thrombospondin-1. Synthetic peptides derived from the corresponding positions in the first and third type I repeats, DGWSPW and GGWGPW, were inactive (Table I).

Because direct binding to immobilized peptides is influenced both by the efficiency of peptide adsorption on plastic and potential changes in conformation induced by this adsorption, the specificity of fibronectin binding to peptide GGWSHW was further examined by inhibition assays (Fig. 2 and Table II). Binding of fibronectin to immobilized GGWSHW was inhibited by soluble GGWSHW, with 50% inhibition at 29 μM. Three hexapeptides containing conservative single amino acid substitutions, GGWSKW, GGYSHW, or GGWSHY were inactive. Thus, the two Trp residues and the His residue are essential. The Ser residue is optimal for binding but not absolutely required (Fig. 2). Substitution with a Thr residue decreased activity ~five-fold. Substitution with an Ala residue also produced an active peptide, with a slightly higher inhibition constant than the Thr analog. The presence of two Gly residues strongly enhanced activity relative to the analog with one Gly residue. Substitution of both Gly residues with Ala or of the first Gly residue with Asp abolished activity. As was found in direct binding, the corresponding hexapeptides from the first and third repeats of thrombospondin were inactive. Peptide 246 was a weak inhibitor, but other peptides that were weakly active in the direct binding assay did not inhibit fibronectin binding to immobilized GGWSHW.

Peptides containing the sequence Trp-Ser-Xaa-Trp were shown previously to bind to heparin and inhibit interactions of several proteins with sulfated glycoconjugates (Guo et al., 1992a,b). To examine the relationship between fibronectin and heparin-binding activities, several of the peptides were tested as inhibitors of thrombospondin binding to heparin. The active peptide GGWSHW and the inactive analog GGWSKW were both weak inhibitors of binding to heparin (IC₅₀ = 100 μM), whereas the peptides GGYSHW and GGWSHY were inactive (IC₅₀ > 400 μM). The peptides GTWSEW and GFWSEW, which were inactive for binding fibronectin

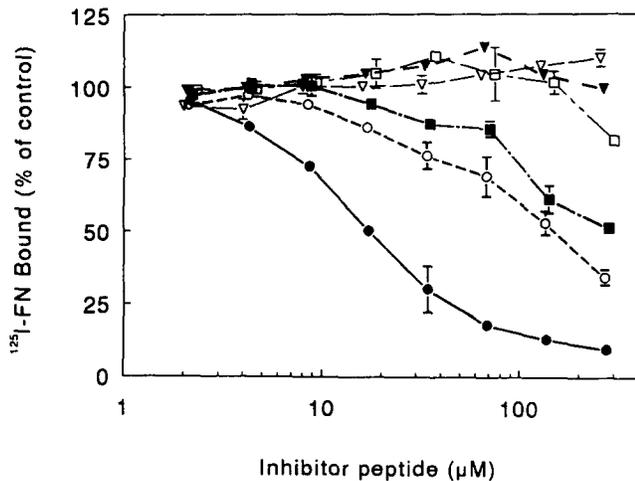


Figure 2. Specificity of inhibition of fibronectin binding to thrombospondin peptide GGWSHW by synthetic peptides. Binding of [125 I]fibronectin (0.5 μ g/ml) to microtiter plate wells coated with 50 μ g/ml of GGWSHW was determined in the presence of the indicated concentrations of the peptides in solution: GGWSHW, ●; GGWTHW, ○; GGWAHW, ■; GWSHW, □; AAWSHW, ▼; and DGWSHW, ▽. Results are mean \pm SD for duplicate determinations and are presented as percent of specific binding determined in the absence of inhibitor.

(Table I), were good inhibitors of thrombospondin binding to heparin with IC_{50} values of 40 and 50 μ M, respectively. Thus, the fibronectin and heparin binding activities are distinct.

Both unlabeled fibronectin and thrombospondin competed for binding of [125 I]fibronectin to the peptide GGWSHW (Fig. 3), but several irrelevant proteins tested (ovalbumin, transferrin, fetuin, goat IgG, and murine laminin) did not ($IC_{50} > 200$ μ g/ml, Fig. 4 and results not shown). Inhibition by thrombospondin suggested that the peptide binds to fibronectin at a site involved in binding of fibronectin to thrombospondin. An alternative explanation for the observed inhibition, that thrombospondin competes by binding to peptide GGWSHW, could not be excluded, because labeled thrombospondin bound to the immobilized peptide, albeit much weaker than fibronectin (results not shown).

To further define the sites involved in the interaction of fibronectin with this peptide and with intact thrombospondin, proteolytic fragments of fibronectin were tested as inhibitors. Of the proteolytic fragments tested, the gelatin-binding domain was the best inhibitor of [125 I]fibronectin binding to immobilized peptide GGWSHW (Fig. 4). Proteolytic fragments containing the cell binding domain were ~ 10 -fold weaker, and fragments containing the fibrin-binding I or carboxyl-terminal heparin-binding domains of fibronectin were inactive. Of the recombinant fibronectin fragments tested, which contained portions of the fibrin I, cell-binding, and heparin-binding domains, only a 33-kD fragment from the cell binding domain was inhibitory. However, a 28-kD fragment containing all but the NH_2 terminal 5-kD of the 33-kD fragment was inactive and a 40-kD fragment with the same amino terminus as the 28-kD fragment was ~ 10 -fold less active. Because the sequence of the 33-kD recombinant fragment is contained in the 120-kD proteolytic fragment of fibronectin, and the latter fragment was a weak inhibitor, this

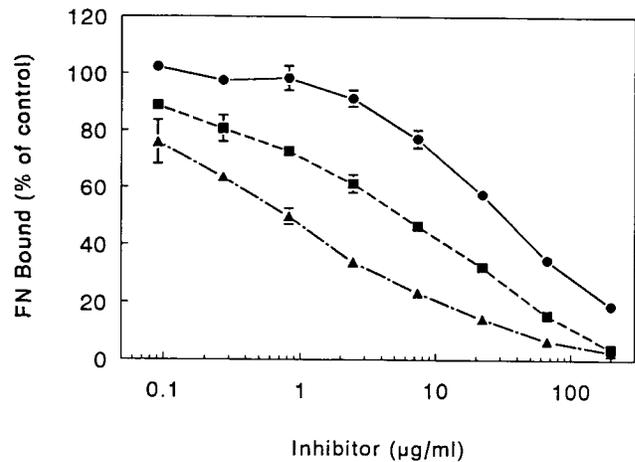


Figure 3. Inhibition of fibronectin binding to thrombospondin peptide GGWSHW by soluble fibronectin, thrombospondin, or peptide GGWSHW. Binding of [125 I]fibronectin (0.5 μ g/ml) to microtiter plate wells coated with 50 μ g/ml of GGWSHW was determined in the presence of the indicated concentrations of the peptide GGWSHW (●), fibronectin (▲), or thrombospondin (■).

may represent a secondary binding site that requires the amino terminal sequence of the 33-kD fragment or is present but cryptic in the 28-kD recombinant fragment. Its activity, however, is less than that of the gelatin binding fragments.

Inhibition by these fragments was not due to binding to the labeled ligand, as none of the fragments used bound significantly to fibronectin in a direct binding assay (results not shown). Iodinated gelatin-binding fragment did not bind to the peptide GGWSHW. However, direct binding of the gelatin-binding domain to peptide GGWSHW was confirmed using a monoclonal antibody that recognized this domain as a detection reagent (Fig. 5). Antibody 191 was chosen because it bound to the gelatin-binding domain of fibronectin

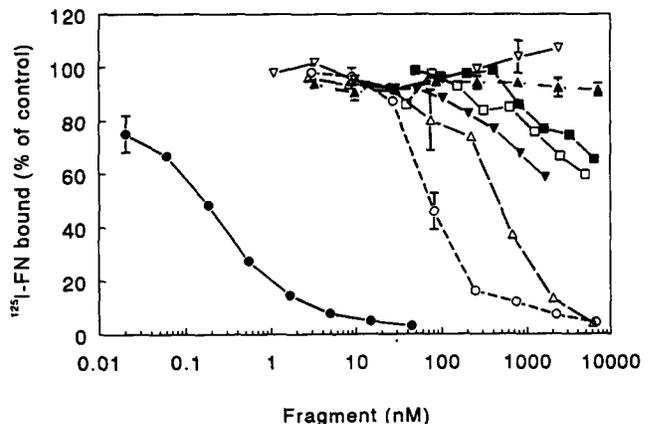


Figure 4. The gelatin-binding domain of fibronectin mediates binding of fibronectin to the thrombospondin peptide GGWSHW. Binding of [125 I]fibronectin (0.5 μ g/ml) to microtiter plate wells coated with 50 μ g/ml of GGWSHW was determined in the presence of the indicated concentrations of proteolytic fragments of fibronectin: intact fibronectin (●), 30-kD gelatin-binding domain (○), 33-kD recombinant cell binding domain (Δ), 28-kD recombinant cell binding domain (\blacktriangle), transferrin (∇), 31-kD fibrin-binding domain (\blacksquare), 40-kD heparin-binding domain (\square), or 120-kD cell-binding domain (\blacktriangledown).

(Newman et al., 1987) but did not inhibit binding of labeled intact fibronectin to the peptide GGWSHW (results not shown). Using this antibody for detection, binding of both intact fibronectin and the gelatin-binding fragment were saturable, with half saturation obtained at 0.7 and 15 $\mu\text{g/ml}$, respectively. No binding was detected using the control fragment from the cell-binding domain of fibronectin.

The gelatin-binding fragment also inhibited fibronectin binding to intact thrombospondin (Fig. 6), indicating that the same domain of fibronectin binds to both the thrombospondin peptide and to intact thrombospondin. Binding of thrombospondin to fibronectin was partially inhibited by peptide 300 (Fig. 6), but the degree of inhibition was variable between experiments and incomplete. The fibrin-binding domain of fibronectin also binds to thrombospondin (Homandberg and Kramer-Bjerke, 1987) and may partially mask effects of the peptide on the interaction of thrombospondin with the gelatin-binding domain of fibronectin.

The peptide GGWSHW also inhibited binding of fibronectin to gelatin (Fig. 7). The inhibition was specific in that the related peptides, GGWSKW, GGYSHW, and GGWSHY, with single amino acid substitutions from the active sequence were inactive. Comparable specificity of inhibition was obtained using immobilized native type I collagen in place of gelatin. The peptide GGWSHW inhibited fibronectin binding to type I collagen with $\text{IC}_{50} = 40 \mu\text{M}$. GGWAHW was a weak inhibitor, whereas WSHWSP, GGWSKW, GGYSHW, GGWSHY, AAWSHW, DGWSHW, and GGWTHW were inactive.

Inhibition of fibronectin binding to gelatin by the peptide GGWSHW was pH dependent (Fig. 8). Inhibition was not detectable at pH 8.5. The potency increased with decreasing pH to a maximum IC_{50} of 1 μM at pH 4.5 to 5.5. At pH 4.0, an inhibition constant could not be determined as fibronectin did not bind to immobilized gelatin. Direct binding of fibronectin to the peptide showed a complex pH-dependence

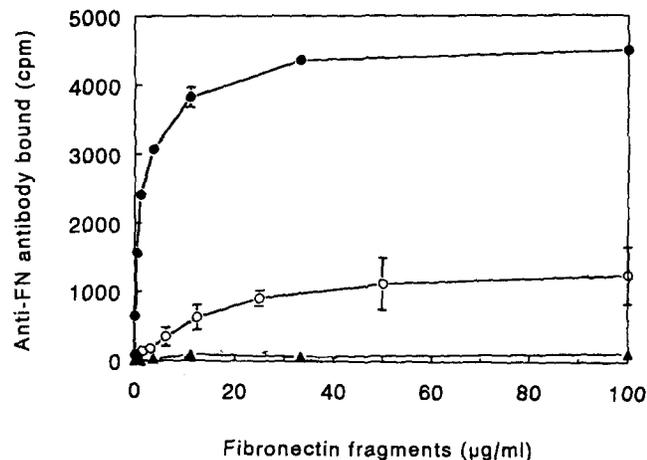


Figure 5. Direct binding of the fibronectin gelatin-binding domain to peptide GGWSHW. Microtiter plate wells coated using 50 $\mu\text{g/ml}$ peptide GGWSHW and blocked by incubation with 1% BSA were incubated with the indicated concentrations of the 30-kD proteolytic gelatin-binding domain of fibronectin (\circ), the 120-kD proteolytic cell binding fragment (\blacktriangle), or intact fibronectin (\bullet). The wells were washed and incubated sequentially with 5 $\mu\text{g/ml}$ antibody 191 and ^{125}I -goat anti-mouse IgG. Bound radioactivity is presented as mean \pm SD for duplicate determinations.

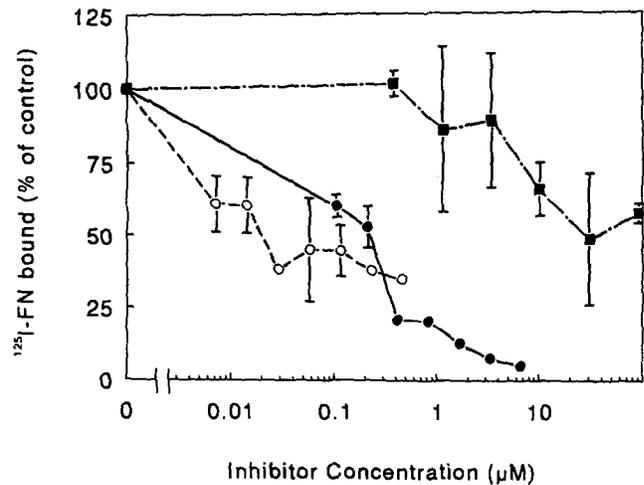


Figure 6. Gelatin binding domain of fibronectin inhibits fibronectin binding to thrombospondin. Microtiter plate wells were coated with 4 μg of thrombospondin in 50 μl of Dulbecco's PBS by incubating for 3 h at 25°C. The wells were emptied and incubated with Dulbecco's PBS containing 1% BSA for 30 min. The wells were emptied and incubated with 0.5 $\mu\text{g/ml}$ [^{125}I]fibronectin in the presence of the indicated concentrations of 30-kD proteolytic gelatin-binding fragment (\bullet), intact fibronectin (\circ), or GGWSHW (\blacksquare).

(data not shown). Binding was strong at pH values less than 6 and from 7.5 to 8 but weaker at pH 6.5 to 7.

The fibronectin-binding peptide GGWSHW was tested for functional effects on the adhesion of human breast carcinoma and melanoma cell lines. The peptide GGWSHW inhibited fibronectin-dependent adhesion of A2058 melanoma cells (Fig. 9 A) or MDA MB435s breast carcinoma cells (Fig. 10 A) to immobilized gelatin. At the concentrations used, the cells adhered weakly to gelatin in the absence of added fi-

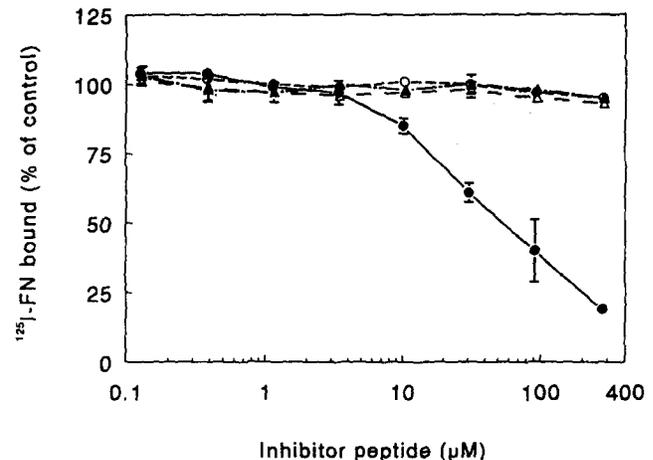


Figure 7. Peptide GGWSHW specifically inhibits fibronectin binding to gelatin. Wells of a polyvinyl chloride microtiter plate were incubated for 3 h at 25°C with 2 $\mu\text{g/ml}$ of gelatin in Dulbecco's PBS. The wells were emptied and incubated with Dulbecco's PBS containing 1% BSA for 30 min. The wells were emptied and incubated with 0.5 $\mu\text{g/ml}$ [^{125}I]fibronectin in the presence of the indicated concentrations of peptide GGWSHW (\bullet), GGWSKW (\circ), GGYSHW (\blacktriangle), or GGWSHY (\triangle) for 2 h at 25°C. The wells were washed four times with PBS, cut from the plate and the bound radioactivity was counted.

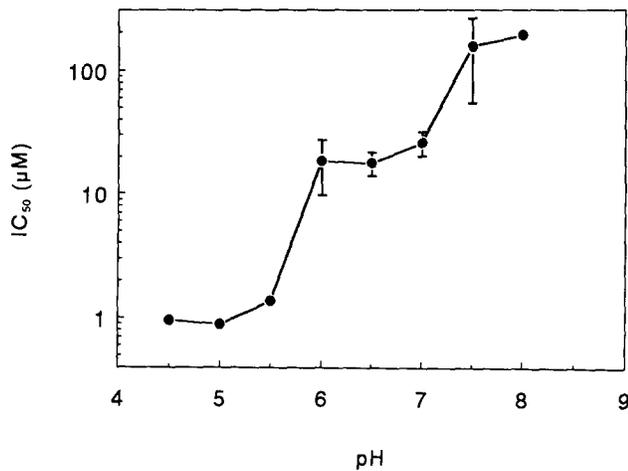


Figure 8. pH-dependence for inhibition by peptide GGWSHW of fibronectin binding to gelatin. Inhibition was determined as in Fig. 7 using a series of buffers of constant ionic strength, 0.15. Error bars indicate \pm SD for determinations of dose for 50% inhibition (IC_{50}) in two or more buffer systems.

fibronectin. The stimulated adhesion in the presence of fibronectin was inhibited in a dose-dependent manner by the peptide GGWSHW. The inhibition was specific in that two homologous peptides that lacked fibronectin binding activity, GGWSKW and WSHWSP, were inactive. Direct adhesion of A2058 cells (Fig. 9 B) or MDA 435s cells (Fig. 10 B) to immobilized fibronectin was not inhibited by the peptide GGWSHW or the control peptides, but adhesion of the breast carcinoma cells to fibronectin was inhibited by the peptide GRGDS (Fig. 10 B). Therefore, interaction of fibronectin with integrin receptors on the breast carcinoma cells is not inhibited by the peptide GGWSHW.

Fibronectin-mediated adhesion of the breast carcinoma cells to native type I collagen was also inhibited by the peptides GGWSHW and GRGDS but not by GGWSKW or WSHWSP (Fig. 11). Direct adhesion to type I collagen was stronger than to gelatin. However, the effects of the peptide GGWSHW were specific for fibronectin stimulated adhesion, as direct adhesion to type I collagen was not inhibited by GGWSHW (results not shown).

Discussion

Fibronectin is an important extracellular matrix component that mediates cell interactions and extracellular matrix assembly during normal development. It also participates in hemostasis and wound healing, and peptides that inhibit fibronectin interactions with cells have been demonstrated to inhibit tumor metastasis in animal models (Humphries et al., 1986). Several synthetic peptides from fibronectin have been shown to inhibit interactions of fibronectin with cell surface integrin receptors (reviewed in Yamada, 1991). Recently, a synthetic peptide from the first type III repeat of fibronectin was demonstrated to inhibit binding of fibronectin to itself during matrix assembly (Morla and Ruoslahti, 1992). The peptides described in the present work define a new class of inhibitors of fibronectin function. The peptide GGWSHW binds specifically to fibronectin. It inhibits interactions of fibronectin with collagen but not with cell surface

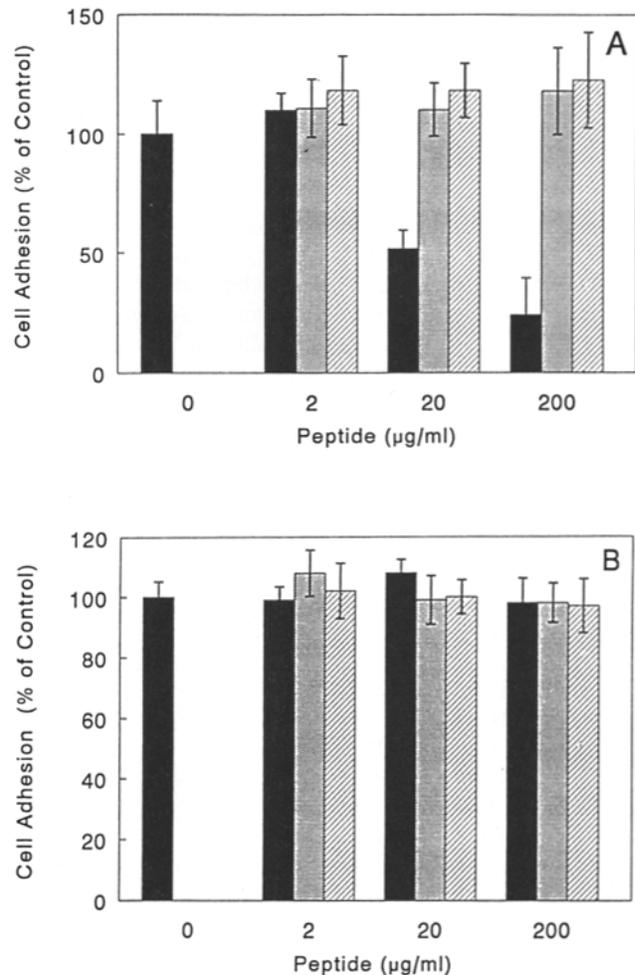


Figure 9. Peptide GGWSHW inhibits fibronectin-dependent cell adhesion to denatured collagen. (A) Adhesion of human melanoma cells, A2058, was determined to plastic coated with 1 µg/ml gelatin in the presence or absence of 1 µg/well of soluble fibronectin and with fibronectin in the presence of the indicated concentrations of the peptide GGWSHW (solid bars) or the control peptides GGWSKW (grey bars) and WSHWSP (striped bars). (B) Direct adhesion of A2058 melanoma cells to plastic coated with 10 µg/ml fibronectin was determined as in A. Data are presented as a percent of control adhesion determined in the absence of peptides (mean \pm SD, $n = 6$).

integrin receptors for fibronectin. Therefore, this class of peptides may have multiple applications in regulation of fibronectin-dependent cell-matrix interactions involved in tumorigenesis, metastasis, wound repair, and hemostasis. The peptide GGWSHW inhibits fibronectin-mediated adhesion of cells to a collagen matrix. We are currently examining the ability of the peptides to inhibit other biological activities of fibronectin in normal and transformed cells.

Interaction of the peptide GGWSHW with fibronectin is specific by several criteria. First, high affinity binding to the immobilized peptide was obtained only with fibronectin, and several unrelated extracellular proteins failed to compete for fibronectin binding to the immobilized peptides. Based on activities of fibronectin fragments, the gelatin-binding domain is the major site for binding to the peptide. A secondary site, however, may also be present in the cell binding do-

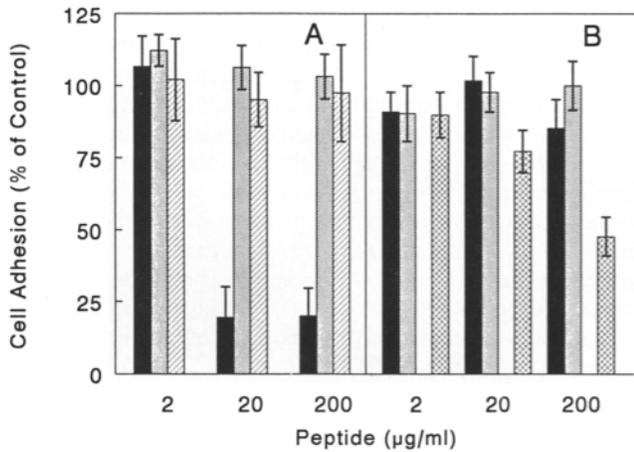


Figure 10. Inhibition of breast carcinoma cell adhesion by peptides. Fibronectin mediated adhesion to gelatin (A) or direct adhesion to fibronectin (B) were determined as in Fig. 8. Inhibition by peptides GGWSHW (solid bars), GGWSKW (grey bars), WSHWSP (striped bars), or GRGDS (hatched bars) is presented as a percent of control (mean \pm SD, $n = 6$).

main. Second, binding is highly sequence specific. The two Trp residues and the His residue are essential, and changing any of the other residues in the hexapeptide substantially decreases its activity. Based on the pH-dependence for inhibiting fibronectin binding to gelatin, protonation of the histidine may be required for this activity but probably is not required for direct binding of the peptide to fibronectin. Finally, the specificity for binding to fibronectin is distinct from that for binding of peptides containing the Trp-Ser-Xaa-Trp consensus sequence to heparin. GGWSHW has weak heparin binding activity, whereas the peptide KRF-KQDGGWSHWSPWSS binds strongly to heparin (Guo et al., 1992a,b). The order of activities is reversed for binding of these two peptides to fibronectin. The peptides GGWSKW, GTWSEW, and GFWSEW have equivalent or better

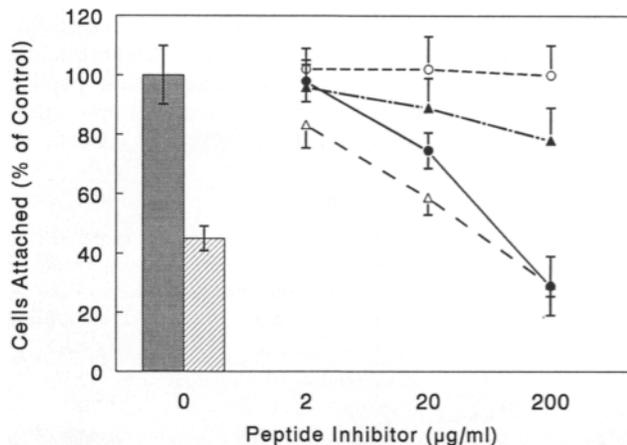


Figure 11. Fibronectin-mediated adhesion of breast carcinoma cells to native type I collagen is inhibited by GGWSHW. Cell attachment was determined to immobilized type I collagen (striped bar) or to type I collagen mediated by soluble fibronectin, alone (grey bar) or in the presence of the indicated concentrations of peptide GGWSHW (●), GGWSKW (○), WSHWSP (▲), or GRGDS (△), mean \pm SD, $n = 6$.

heparin-binding activities than GGWSHW but are inactive for inhibiting binding of fibronectin to the immobilized peptide GGWSHW or to gelatin.

The peptide identified here is probably not the only site for interaction of thrombospondin with fibronectin, and at least two domains of thrombospondin have been shown to bind to fibronectin (Dardik and Lahav, 1989). Proteolytic fragments from the amino terminus and the 70-kD core of thrombospondin both bound to intact fibronectin. Binding to thrombospondin may also be mediated by more than one site on fibronectin. There is direct evidence for a role of the fibrin-binding domain of fibronectin in its interaction with thrombospondin (Homandberg and Kramer-Bjerke, 1987). However, Gelder and Brown (1987) reported that thrombospondin can inhibit interactions of fibronectin with gelatin. Both whole thrombospondin isolated from platelets and an unidentified component of human plasma inhibited agglutination of mixtures of gelatin-coated latex beads and fibronectin-coated beads. The domains or amino acid sequences responsible for this activity were not examined. The present results, that the gelatin-binding domain of fibronectin inhibits binding of fibronectin to both thrombospondin and the thrombospondin peptide GGWSHW and that this peptide specifically inhibits thrombospondin binding to fibronectin, are consistent with the latter data.

The ability of a sequence in thrombospondin to inhibit fibronectin mediated cell adhesion to a collagen matrix suggests a mechanism for the anti-adhesive activity of thrombospondin (reviewed in Sage and Bornstein, 1991). Thrombospondin disrupts focal contact adhesions of endothelial cells attached to a fibronectin matrix (Murphy-Ullrich and Höök, 1989). The mechanism of this effect is unknown, but inhibition of the activity of thrombospondin by sulfated polysaccharides indicated that a heparin-binding domain of thrombospondin was involved. However, both fibronectin and heparin inhibited the binding to endothelial cells of intact thrombospondin or the 70-kD fragment (Dardik and Lahav, 1991), suggesting that the heparin and fibronectin binding sites in the 70-kD fragment are proximal. These data are consistent with the binding activities of isolated peptides from the type I repeats of thrombospondin in that the fibronectin binding sequence is contained within a peptide that binds with high affinity to heparin. We have previously shown that each of the three type I repeats of thrombospondin contain heparin-binding sequences with the consensus sequence Trp-Ser-Xaa-Trp-Ser (Guo et al., 1992a,b). The present results identify the fibronectin-binding sequence Gly-Gly-Trp-Ser-His-Trp, which occurs only in the second Type I repeat. The first Trp residue in the Trp-Ser-Pro-Trp-Ser sequence from this repeat corresponds to the carboxyl-terminal Trp residue in the fibronectin binding sequence. Because the thrombospondin peptide GGWSHW is anti-adhesive for fibronectin mediated cell adhesion to gelatin or type II collagen, the sequence could account for some of the inhibitory effects of thrombospondin on cell adhesion. The overlap with the heparin binding sequence would account for sensitivity of the anti-adhesive activity to heparin (Murphy-Ullrich and Höök, 1989).

The availability of this sequence in the intact protein and in the presence of heparin, however, remains to be addressed. Because the hexapeptide GGWSHW bound fibronectin better than the larger peptides containing the same se-

quence or intact thrombospondin, this binding site appears to be partially cryptic in thrombospondin. The lower activity of peptide 246 relative to GGWSHW suggests that neighboring residues in the larger peptide could directly inhibit binding of the active sequence to fibronectin. Further studies will be required, however, to examine effects of neighboring sequences on the conformation of the active sequence in this family of peptides and to determine the accessibility and conformation of this sequence in native thrombospondin. Previous studies suggested that the fibronectin binding site in the 70-kD fragment of endothelial cell thrombospondin is cryptic in the presence of divalent cations (Dardik and Lahav, 1989). However, the ability of antisera to synthetic peptides from the type I repeats of thrombospondin to bind to intact thrombospondin demonstrates that at least portions of the type I repeats are accessible in the native protein (Prater et al., 1991; Tuszynski et al., 1992). The amino-terminal fibrin-binding domain of fibronectin is a second site that mediates binding to thrombospondin. Therefore, it may be difficult to efficiently inhibit binding of the intact proteins using only an inhibitor of interaction with the gelatin-binding domain.

The high degree of conservation of the fibronectin-binding hexapeptide in the thrombospondin gene family suggests a functional role of this sequence (Table III). The sequence Gly-Gly-Trp-Ser-His-Trp is completely conserved in the second type I repeats of mouse and human thrombospondin-1 and in thrombospondin-2 from mouse and chicken (Laherty et al., 1992). However, it is not present in any of the six type I repeats in the neuronal homolog of thrombospondin, F-spondin (Klar et al., 1992). The heparin-binding consensus sequence Trp-Ser-Xaa-Trp-Ser is conserved in complement components and in many members of the cytokine receptor and transforming growth factor β superfamilies (Bazan, 1990; Wharton, 1991; Guo et al., 1992a,b). The sequence Gly-Gly-Trp-Ser-His-Trp, however, does not occur in these proteins. A search of the Swiss-Prot data base using the search sequence Gly-Gly-Trp-Ser-His-Trp yielded several other proteins with similar sequences (Table III). Epstein-Barr virus ribonucleotide reductase contains the most homologous sequence, Gly-Gly-Trp-Phe-His-Trp, and the flanking sequences contain three additional matches with the sequence of the second thrombospondin type I repeat, including

Table III. Amino Acid Sequence Homologies with the Fibronectin-binding Sequence from Thrombospondin

Source	Sequence
Human <i>THBS1</i>	KRFKQDGGWSHWSPWSSC
mouse <i>Thbs1</i>	KRFKQDGGWSHWSPWSSC
mouse <i>Thbs2</i>	TRIRQGGWSHWSPWSSC
chicken <i>Thbs 2</i>	HRIRQDGGWSHWSPWSSC
EBV ribonucleotide reductase	KQSKYAGGWFHHDWAGC
chicken AchR α	WVMKDYRGWKHWVYYACC
<i>T. californica</i> AchR α	WVMKDYRGWKHWVYYTCC
rat AchR α	WVIRKARGWKHWVFYSCC
mouse AchR α	WVIRKARGWKHWVFYSCC
bovine AchR α	WVIRKESRGWKHWVFYACC

○ ○ * **

Sequence identity with the thrombospondin sequence is indicated by bold residues. Residues in acetylcholine receptor α subunit (AchR α) identified by cross-linking to receptor ligands (Karlin, 1991) are indicated by *. Residues implicated in α -bungarotoxin binding (Barchan et al., 1992) are indicated by ○.

the third Trp residue and the basic residues involved in heparin binding. This match may be fortuitous, however, as this homologous sequence is not conserved in other viral or eukaryotic ribonucleotide reductase genes (Nikas et al., 1986). A potentially more interesting homology is with the sequence Gly-Trp-Lys-His-Trp in the α -subunit of the nicotinic acetylcholine receptor, which is highly conserved among all vertebrate acetylcholine receptor sequences. Based on affinity labeling, a peptide containing this sequence plays a role in acetylcholine binding to the receptor. Three residues adjacent to the homologous sequence, Tyr 190, Cys 192 and Cys 193, have been implicated in binding acetylcholine analogs (reviewed in Karlin, 1991). The latter Cys residue is conserved at the same relative position in the thrombospondin genes. Recombinant active site fragments from the acetylcholine receptor containing the homologous sequence bind α -neurotoxins with relatively high affinity (Ohana and Gershoni, 1990). The second Trp residue, Trp 187, may participate in binding of α -bungarotoxin to the receptor (Barchan et al., 1992).

Site directed mutagenesis of two cytokine receptors also identified the WSXW motif as a potential "binding" sequence (Miyazaki et al., 1991; Yoshimura et al., 1992). The ligands that interact with this site were not identified, but loss of receptor processing or activity after mutation of the WSXW consensus suggests that cellular factors involved in protein folding, intracellular processing, or signal transduction are potential ligands. Using synthetic peptides, we have identified two ligands for this sequence: heparin and related sulfated glycoconjugates for WSXW and fibronectin for the subset with X = His. We propose that there may be other ligands yet to be identified for other subsets of this consensus sequence.

The type I and type II repeats from the collagen-binding domain of fibronectin have been conserved in several other proteins (Peterson et al., 1989). If the sequence or conformational epitope responsible for binding of the peptide GGWSHW to fibronectin is conserved in these proteins, the thrombospondin peptide may be a useful inhibitor of their functions. The thrombospondin peptides are therefore being tested as inhibitors of the activities of other proteins that contain domains homologous to the fibronectin gelatin-binding domain. Preliminary experiments indicate that the peptide GGWSHW inhibits gelatinase activity secreted by corneal endothelial cells (J. M. Sipes, H. C. Krutzsch, and D. D. Roberts, unpublished results).

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