

1999

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Recommended Citation

Hocking D.C., Sottile J., Reho T., Fässler R., McKeown-Longo P.J. (1999) Inhibition of fibronectin matrix assembly by the heparin-binding domain of vitronectin. *Journal of Biological Chemistry* 274: 27257-27264. doi: 10.1074/jbc.274.38.27257

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Inhibition of Fibronectin Matrix Assembly by the Heparin-binding Domain of Vitronectin*

(Received for publication, November 23, 1998, and in revised form, April 6, 1999)

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The deposition of fibronectin into the extracellular matrix is an integrin-dependent, multistep process that is tightly regulated in order to ensure controlled matrix deposition. Reduced fibronectin deposition has been associated with altered embryonic development, tumor cell invasion, and abnormal wound repair. In one of the initial steps of fibronectin matrix assembly, the amino-terminal region of fibronectin binds to cell surface receptors, termed matrix assembly sites. The present study was undertaken to investigate the role of extracellular signals in the regulation of fibronectin deposition. Our data indicate that the interaction of cells with the extracellular glycoprotein, vitronectin, specifically inhibits matrix assembly site expression and fibronectin deposition. The region of vitronectin responsible for the inhibition of fibronectin deposition was localized to the heparin-binding domain. Vitronectin's heparin-binding domain inhibited both β_1 and non- β_1 integrin-dependent matrix assembly site expression and could be overcome by treatment of cells with lysophosphatidic acid, an agent that promotes actin polymerization. The interaction of cells with the heparin-binding domain of vitronectin resulted in changes in actin microfilament organization and the subcellular distribution of the actin-associated proteins α -actinin and talin. These data suggest a mechanism whereby the heparin-binding domain of vitronectin regulates the deposition of fibronectin into the extracellular matrix through alterations in the organization of the actin cytoskeleton.

The deposition of fibronectin into the extracellular matrix is a dynamic, multistep process that is normally tightly regulated in order to ensure controlled matrix deposition. In certain disease states, including pulmonary fibrosis and atherosclerosis, loss of this regulation gives rise to either excess or inappropriate fibronectin deposition (1). In addition, reduced fibronectin deposition has been associated with altered embryonic development, tumor cell invasion, and abnormal

wound repair (1). The mechanisms that control the rate and extent of fibronectin deposition are only partially understood.

Adherent cells polymerize an insoluble fibronectin matrix by assembling cell- or plasma-derived soluble fibronectin into insoluble fibrils (2). In one of the initial steps of matrix assembly, cell surfaces bind the amino-terminal region of fibronectin in a reversible and saturable manner (3, 4). Subsequent homophilic binding interactions are thought to promote the polymerization of the fibronectin molecule into an insoluble matrix (5–9) and allow for the regeneration of the cell surface amino-terminal binding site (2). The binding of the amino terminus of fibronectin to cell surface receptors, termed matrix assembly sites (3), is mediated by the first five type I repeats of fibronectin (4, 10). The molecule(s) that mediates the binding of the amino terminus of fibronectin to cell surfaces has not been definitively identified. It has been proposed that the III₁ module of fibronectin (8, 11), large molecule mass molecules (12), and the fibronectin-binding $\alpha_5\beta_1$ integrin (13–15) interact with the amino terminus of fibronectin on cell surfaces.

Expression of cell surface matrix assembly sites may be rapidly up- and down-regulated by protein kinase C activation, cyclic AMP levels, and 1-oleoyl lysophosphatidic acid (LPA)¹ treatment (16–18). Activators of protein kinase C, which effect both stress fiber and focal contact formation (19), enhance fibronectin matrix assembly (17). Conversely, increasing intracellular cAMP levels, which disrupt actin stress fibers and cause cell retraction (20), inhibit matrix assembly (16). In addition, studies have demonstrated loss of matrix assembly sites (21) and decreased fibronectin deposition (22) upon disruption of the actin cytoskeleton with cytochalasin D. More recent studies have defined a role for Rho-mediated contractility in the expression of matrix assembly sites and fibronectin deposition (18, 23, 24). Increasing Rho-stimulated contraction through treatment of cells with either LPA (18, 23) or nocodazole (24) or by microinjection of recombinant, constitutively active Rho (23) results in an increase in amino-terminal fibronectin fragment binding and enhanced fibronectin deposition. Taken together, these observations indicate that matrix assembly site expression and fibronectin deposition are regulated, in part, by actin stress fiber formation and the contractile state of the cell.

Although it has been well documented that decreased fibronectin matrix assembly occurs in most transformed and tumor-derived cell lines, little is known about the extracellular signals that regulate matrix assembly activity in normal fibroblasts. Earlier studies have demonstrated that cells adherent

* This work was supported by National Institutes of Health Grants CA-69612 and CA-58626 (to P. J. M.-L.) and HL-50549 (to J. S.) and American Heart Association, New York State Affiliate, Grants 950210 (to D. H.) and 950318 (to J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: LPA, 1-oleoyl lysophosphatidic acid; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; FnIII, recombinant human fibronectin type III module; Vn, vitronectin.

to vitronectin exhibit decreased levels of cell surface matrix assembly sites compared with fibronectin-adherent cells (21, 25–27). These data suggest the possibility that the interaction of cells with vitronectin generates inhibitory signals that regulate matrix assembly site expression. In the present study, we demonstrate that the interaction of cells with vitronectin inhibits matrix assembly site expression and fibronectin deposition. The region of vitronectin responsible for the inhibition of fibronectin matrix assembly was localized to the heparin-binding domain. The inhibitory effect of vitronectin's heparin-binding domain was independent of the integrin receptor used to assemble the fibronectin matrix. Vitronectin-mediated inhibition of matrix assembly site expression could be overcome by treatment of cells with LPA, an agent that promotes actin polymerization (18, 24). Moreover, the interaction of fibronectin-adherent cells with the heparin-binding domain of vitronectin resulted in changes in actin microfilament organization and the subcellular distribution of the actin-associated proteins, α -actinin, and talin. These data suggest a mechanism whereby the heparin-binding domain of vitronectin down-regulates fibronectin matrix assembly through alterations in the organization of the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Gel electrophoresis supplies were from Bio-Rad. Unless otherwise indicated, chemicals were obtained from Sigma. The polyclonal anti-fibronectin antibody was prepared by as described previously (28). The following monoclonal antibodies were purchased: anti-integrin $\alpha_v\beta_3$ (LM609), integrin subunit β_5 (P1F6), α -actinin, and talin from Chemicon International (Temecula, CA); anti-vinculin antibody from Sigma.

Cell Culture—Human foreskin fibroblasts, A1-Fs, were a gift from Dr. Lynn Allen-Hoffmann (University of Wisconsin, Madison, WI). A1-Fs were cultured in Dulbecco's modified eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sterile Systems, Logan, UT). GD25 and GD25 β_1 cells were cultured as described previously (29).

Purification of Plasma Proteins and Fragments—Human plasma fibronectin was purified from a fibronectin- and fibrinogen-rich by-product of Factor VIII production by ion exchange chromatography on DEAE-cellulose (Amersham Pharmacia Biotech) as described previously (30). The 70-kDa amino-terminal fragment of fibronectin was generated by limited digestion of intact fibronectin with cathepsin D, followed by gelatin affinity chromatography as described previously (30).

Vitronectin was purified from fibronectin- and fibrinogen-depleted human plasma by heparin-Sepharose (Amersham Pharmacia Biotech) affinity chromatography according to the method of Yatohgo *et al.* (31). The 40-kDa cell-binding fragment of vitronectin was generated by acid cleavage of intact vitronectin (32). Purified vitronectin was dissolved in 70% formic acid and incubated at 37 °C for 46 h. The digested vitronectin was diluted 1:10 with water, lyophilized, and reconstituted in 50 mM Tris, pH 7.6. The 40-kDa cell-binding fragment of vitronectin was separated from heparin-binding fragments by exclusion on a heparin-Sepharose affinity column. Vitronectin fragments that bound to the heparin-Sepharose were eluted with 0.5 M NaCl in 25 mM phosphate buffer, pH 7.4. Both the cell-binding and heparin-binding fragments were dialyzed extensively against PBS prior to use. Purity of protein preparations was assessed by SDS-polyacrylamide gel electrophoresis, and proteins were frozen at -80 °C until use.

Rat tail collagen type I was purchased from Beckton Dickinson. Laminin (purified from EHS tumors) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Thrombospondin was purified from platelet releasate as described previously (72) and was a gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI). Fibrinogen was purified from human plasma (33) and was a gift of Dr. John Kaplan (Albany Medical College).

Purification of Recombinant Proteins—Recombinant human fibronectin III_{9,10} (FnIII_{9,10}) was expressed in BL21 (DE3) bacteria as a fusion protein with glutathione S-transferase (GST) as described previously (9). FnIII_{9,10} was separated from GST by digestion with trypsin followed by chromatography over glutathione-agarose (9).

Polymerase chain reaction (PCR) was used to amplify human fibronectin cDNA encoding the 12th and 13th type III modules of fibronectin (FnIII_{12,13}) (bases 5081–5623). This DNA encodes amino acids

Ala-1689 through Thr-1869, which represents the major heparin-binding region of fibronectin (34). Bases are numbered from the A in the codon for the first amino acid of the mature protein (EMBL accession number X02761), and amino acids are numbered from the amino-terminal pyroglutamic acid (35). PCR was also used to amplify human vitronectin cDNA encoding the heparin-binding domain of vitronectin (Vn_{HBD}) (bases 1138–1258) (36, 37). This DNA encodes amino acids from Ala-341 through Ala-380 (38, 39). The sense primers for the FnIII_{12,13} construct (5'-CCGGATCCGCTATTCCTGCACCAACTGAC) and the Vn_{HBD} construct (5'-CCCGGATCCGCACCCCGCCCTCTT-G) were synthesized with a *Bam*HI site (shown in boldface type) at the 5'-end. The antisense primers for the FnIII_{12,13} construct (5'-CCCGA-ATTCCCTATAGTGGAGGC GTCGATGACCA) and the Vn_{HBD} construct (5'-CCCGAATTCCCTAGGCGCGGGATGGCCGGCG) were synthesized with an *Eco*RI site (shown in boldface type) at the 5'-end. Underlined bases introduce a stop codon after the last base in the sequence to be amplified. PCR was performed according to established procedures (40), using human full-length fibronectin cDNA, pFH100 (a gift from Dr. Jean Thiery, Paris, France) or vitronectin cDNA (a gift from Dr. Erkki Ruoslahti, Burnham Institute, La Jolla, CA) as a template. Following restriction enzyme digestion, the PCR-amplified DNA was cloned into the bacterial expression vector pGEX-2T (Amersham Pharmacia Biotech) and transfected into BL21 (DE3) bacteria using standard procedures (40). PCR-amplified DNA was sequenced to confirm that no base changes had been introduced during amplification of the DNA. Fusion proteins were isolated by passing bacterial lysates over glutathione-agarose as described previously (8) and were dialyzed extensively against PBS prior to use. Both GST-FnIII_{12,13} and GST-Vn_{HBD} bound avidly to heparin-Sepharose (data not shown).

Recombinant vitronectins were produced using a baculovirus expression system. Nonmutant and mutant vitronectins were PCR-amplified using the sense primer 5'-GGCTACCGTTCACAACGA and the antisense primer 5'-GGGTCTAGACTACAGATGGCCAGGACTGG. The Vn_{RGE} mutant was produced using recombinant PCR (41). The two mutagenic primers were 5'-GCAAGCCCCAAGTGACTCGCGGGGAG-GTGTTCACTATGCCGAGGATGAGT (sense) and 5'-ACTCATCCTC-CGGCATAAGTGAACACCTCCCCGCGAGTCACTTGGGGCTTGC (anti-sense). The mutated bases are shown in boldface type. The two outer primers used were the same as those used to amplify nonmutant vitronectin. PCR-amplified DNA was cloned into the baculovirus expression vector, pVL1392 (Pharmlingen), using the restriction enzymes *Not*I and *Xba*I.

Recombinant viruses were generated according to established procedures (42, 43). Viral stocks were prepared using SF21 cells grown in serum-free SF900II medium (Life Technologies, Inc.). Supernatants from infected cells were collected 72 h postinfection. To purify the recombinant vitronectins, supernatants were treated with 8 M urea for 2 h at 20 °C and passed over columns of heparin-Sepharose (Amersham Pharmacia Biotech). Resins were washed with 130 mM NaCl, 4 M urea in 25 mM phosphate, pH 6.5, and bound proteins were eluted with 0.5 M NaCl, 4 M urea in 50 mM phosphate buffer, pH 8. The recombinant vitronectins were dialyzed extensively against PBS. Purity of the recombinant protein preparations was assessed by SDS-polyacrylamide gel electrophoresis, and proteins were frozen at -80 °C until use.

Cell Binding Assays—The 70-kDa fibronectin fragment was iodinated with 1.0 mCi of Na¹²⁵I (NEN Life Science Products) using chloramine T as described previously (3). Fibronectin, laminin, vitronectin, thrombospondin, anti-integrin antibodies, and FnIII_{9,10} were diluted to 10 μ g/ml in PBS and coated onto 24-well tissue plates (Corning/Costar) for 3 h at 37 °C. Thrombospondin was reduced with 20 mM dithiothreitol for 30 min at 20 °C prior to use (44). Fibrinogen was coated onto culture plates at 100 μ g/ml in PBS. Collagen was coated onto culture plates in 0.02 N acetic acid at 50 μ g/ml at 4 °C overnight. Protein-coated wells were washed three times with PBS before use. To minimize endogenous fibronectin levels during experimental procedures, cells were washed three times with serum-free DMEM and pretreated for 3.5 h with cycloheximide (20 μ g/ml) (30) in DMEM containing ITS+2 (Sigma) as described previously (15). Cells were seeded at 10⁵ cells/well in DMEM/ITS+2 with 20 μ g/ml cycloheximide. Following an overnight incubation, cells were washed three times with serum-free DMEM and incubated for 1 h with 0.5 \times 10⁶ cpm/ml ¹²⁵I-labeled 70-kDa fragment (~20 ng/well) in DMEM containing 0.2% bovine serum albumin (45). Cell adhesion was quantitated in parallel wells by staining with 0.5% crystal violet as described previously (15).

Immunofluorescence Microscopy—For fibronectin matrix assembly assays, non-cycloheximide-treated fibroblasts were seeded onto fibronectin-coated coverslips in 12-well cluster dishes at 10⁵ cells/well in DMEM/ITS+2 in the absence or presence of 1.7 μ M GST/Vn_{HBD}, GST/

FnIII_{12,13}, or 0.75 μ M recombinant vitronectin and incubated overnight at 37 °C. Cells were fixed and permeabilized, and fibronectin fibrils were visualized using a polyclonal anti-fibronectin antibody followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Cappel). Cells were examined using an Olympus BX60 microscope equipped with epifluorescence and photographed using a spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

To assay changes in actin distribution, confluent fibroblasts were washed with serum-free media and incubated overnight in serum-free DMEM. Cells were detached with trypsin/EDTA, washed, and seeded onto fibronectin coverslips in 12-well cluster dishes at 1.5×10^4 cells/well in DMEM/ITS+2 in the absence or presence of 3.4 μ M of GST/Vn_{HBD}, GST/FnIII_{12,13}, or GST. Following an overnight incubation at 37 °C, actin filaments were visualized by staining fixed and permeabilized cells with fluorescein isothiocyanate-phalloidin (Molecular Probes, Inc., Eugene, OR). Vinculin was visualized using an anti-vinculin antibody (Sigma) followed by a Texas Red-conjugated goat anti-mouse secondary antibody (Cappel).

Cellular Localization of Actin-associated Proteins—To assay changes in the cellular distribution of α -actinin, talin, and vinculin, serum-starved, non-cycloheximide-treated fibroblast cells were fractionated into digitonin-soluble and -insoluble (cytoskeletal-associated) fractions (46). Cells were seeded at 4×10^5 cell/well into fibronectin-coated six-well tissue culture plates in the absence or presence of 1.7×10^{-6} M GST/Vn_{HBD} or GST/FnIII_{12,13}. Following an overnight incubation at 37 °C, cells were washed three times with cold PBS and incubated for 5 min on ice with 0.1% digitonin in 50 mM Hepes, pH 6.9, containing 1 mM MgCl₂, 1 mM EDTA, 1 mM EDTA, and inhibitors (10 mM sodium pyrophosphate, 50 mM sodium fluoride, 25 mM β -glycerophosphate, 25 μ g/ml leupeptin, 25 μ g/ml aprotinin, 0.5 mM sodium orthovanadate, 1 mM H₂O₂, 0.5 mg/ml soybean trypsin inhibitor, and 2 mM phenylmethylsulfonyl fluoride). Following removal of the digitonin-soluble fractions, cells were washed two times with PBS and incubated on ice with radioimmune precipitation buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate in 50 mM Tris, pH 7.6, with 150 mM NaCl and inhibitors). Lysates were clarified by centrifugation at $15,000 \times g$ for 10 min at 4 °C. Supernatants were assayed for protein concentration using bicinchoninic acid (BCA) reagents (Pierce) according to the manufacturer's instructions. Samples (10 μ g) were analyzed under nonreducing conditions by SDS-polyacrylamide gel electrophoresis and immunoblotting as described previously (8). Immunoblots were developed using enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

RESULTS

Effect of Adhesive Substrates on Matrix Assembly Site Expression—The polymerization of a fibronectin matrix is a cell-dependent process that is regulated by the expression of specific matrix assembly sites on the surface of substrate-attached cells (2). These sites have been identified by radioligand binding assays using either ¹²⁵I-labeled 70-kDa or ¹²⁵I-labeled 27-kDa fragments derived from the amino terminus of fibronectin (3, 4). Previous studies have demonstrated that cells adherent to vitronectin do not express matrix assembly sites (21, 25, 26). To examine further the effect of extracellular matrix molecules on the expression of matrix assembly sites, cycloheximide-pretreated fibroblasts were allowed to adhere and spread on tissue culture wells coated with various adhesive proteins. Matrix assembly site expression on these cells was then measured in a 1-h binding assay using ¹²⁵I-labeled 70-kDa fragment. Previous studies have identified a homophilic binding site for the amino terminus of fibronectin within the III₁ module (8, 11). Therefore, fibroblasts were also seeded onto the RGD-containing III_{9,10} modules of fibronectin, which mediate cell adhesion (9) but do not contain III₁. As shown in Fig. 1A, cells adherent to vitronectin bound significantly less ¹²⁵I-labeled 70-kDa fragment than cells adherent to either laminin, fibronectin, FnIII_{9,10}, collagen, thrombospondin, or fibrinogen. These results suggest that among these adhesive proteins, vitronectin is distinct in its ability to down-regulate matrix assembly sites.

Previous studies have shown that the expression of matrix assembly sites on fibroblast monolayers is rapidly up-regulated either by activation of protein kinase C with phorbol esters (47)

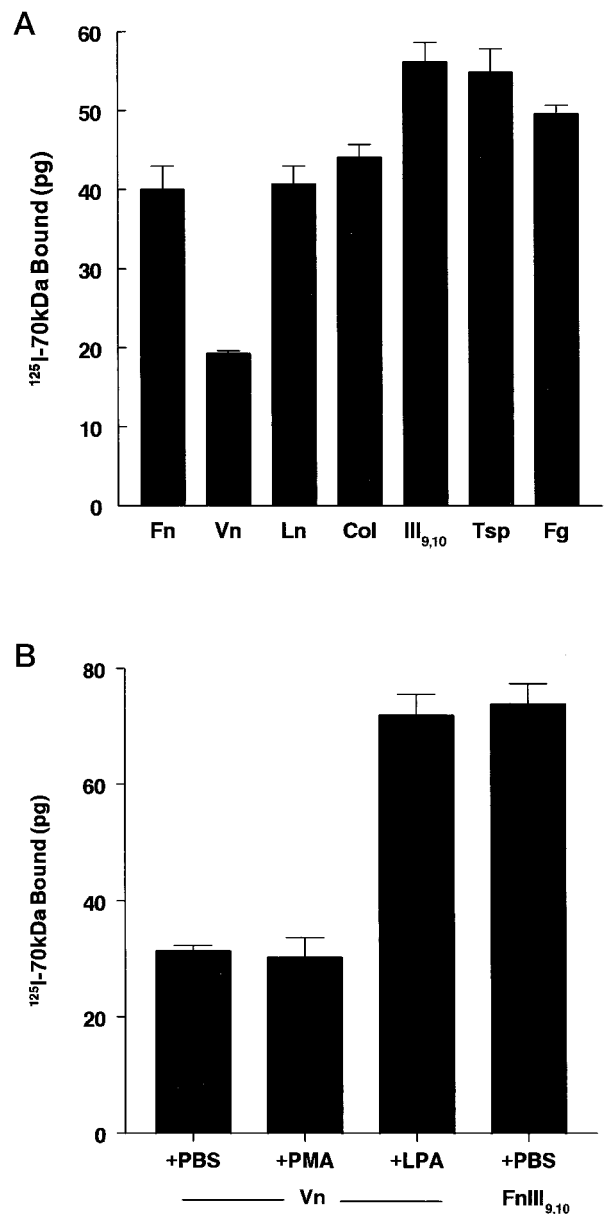


FIG. 1. Effect of adhesive substrates on 70-kDa binding site expression. A, cycloheximide-pretreated fibroblasts were seeded onto tissue culture wells precoated with vitronectin (Vn), laminin (Ln), fibronectin (Fn), recombinant III₉ and III₁₀ modules of fibronectin (FnIII_{9,10}), collagen (Col), thrombospondin (Tsp), or fibrinogen (Fg) as described under "Experimental Procedures." Cells were allowed to adhere and spread for 15 h at 37 °C. ¹²⁵I-labeled 70-kDa fragment binding assays were performed as described under "Experimental Procedures." Similar numbers of cells adhered to each substrate as assessed by staining parallel wells with crystal violet (data not shown). B, cells were seeded onto vitronectin (Vn) or FnIII_{9,10} and left untreated (+PBS) or incubated with 100 nM PMA (+PMA) or 2 μ M LPA (+LPA) for 1 h prior to assay. Data are presented as the amount of ¹²⁵I-labeled 70-kDa fragment bound per well \pm S.E. and represent one of three experiments done in triplicate.

or by increasing Rho-stimulated contractility through treatment with either LPA (18, 23) or nocodazole (24). To determine whether matrix assembly site expression on vitronectin-adherent cells could be similarly increased, cycloheximide-pretreated, vitronectin-adherent cells were incubated with either PMA or LPA for 1 h, and the level of ¹²⁵I-labeled 70-kDa fragment binding was assessed. Treatment of vitronectin-adherent cells with 2 μ M LPA resulted in a 2-fold increase in ¹²⁵I-labeled 70-kDa fragment binding to levels similar to that

observed on cells adherent to FnIII_{9,10} (Fig. 1B). In contrast, treatment of vitronectin-adherent cells with PMA had no effect on ¹²⁵I-labeled 70-kDa fragment binding (Fig. 1B). These data indicate that matrix assembly site expression on vitronectin-adherent cells can be up-regulated upon stimulation of Rho-mediated signaling pathways.

Inhibition of 70-kDa Fragment Binding by Heparin-binding Fragments of Vitronectin—The ability of LPA to stimulate ¹²⁵I-labeled 70-kDa fragment binding to vitronectin-adherent cells suggests the possibility that the decreased basal level of matrix assembly site expression on vitronectin-adherent cells may be due to inhibitory signals generated upon cell adhesion to vitronectin. Vitronectin is a modular glycoprotein that contains domains that mediate binding to both cells and heparin (32). Cell adhesion to vitronectin is mediated by the RGD sequence located in the amino-terminal connecting sequence (32, 48–50), while vitronectin's heparin-binding activity has been localized to a series of basic amino acid residues in the carboxyl-terminal heparin-binding domain (32, 51). To determine which regions of vitronectin are important for regulating matrix assembly site expression, intact vitronectin was subjected to limited proteolysis with formic acid. Cell- and heparin-binding fragments were then separated by heparin affinity chromatography (32). Cycloheximide-pretreated fibroblasts were seeded onto tissue culture wells coated with either the 40-kDa cell-binding fragment of vitronectin or intact fibronectin in the absence and presence of various concentrations of heparin-binding vitronectin fragments. As shown in Fig. 2A, cells adherent to the cell-binding fragment of vitronectin bound ¹²⁵I-labeled 70-kDa fragment to a similar extent as cells adherent to intact fibronectin, suggesting that ligation of vitronectin binding integrins is permissive for the expression of matrix assembly sites. The addition of increasing amounts of heparin-binding fragments of vitronectin to cells adherent to either the cell-binding fragment of vitronectin or intact fibronectin resulted in a dose-dependent decrease in ¹²⁵I-labeled 70-kDa fragment binding (Fig. 2A). At the highest concentrations of heparin-binding fragment tested, ¹²⁵I-labeled 70-kDa fragment binding to cells decreased to that observed on cells adherent to intact vitronectin (Fig. 2A).

Adhesion of fibroblasts to vitronectin is mediated primarily by the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (52, 53). To determine whether ligation of either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins results in the expression of matrix assembly sites, cycloheximide-pretreated cells were seeded onto tissue culture wells coated with the vitronectin integrin receptor-specific peptide, GPenGRGDSP (50), or the anti- $\alpha_v\beta_3$ (LM609) and $\alpha_v\beta_5$ (P1F6) monoclonal antibodies in the absence or presence of 20 μ g/ml heparin-binding fragments. Similar numbers of cells adhered to all wells, as assessed by crystal violet staining (data not shown). As shown in Fig. 2B, ligation of integrin receptor $\alpha_v\beta_3$ or $\alpha_v\beta_5$ resulted in levels of ¹²⁵I-labeled 70-kDa fragment binding that were similar to the level observed on cells adherent to intact fibronectin. The addition of vitronectin heparin-binding fragments decreased ¹²⁵I-labeled 70-kDa fragment binding to cells adherent to either GPenGRGDSP or to the anti- $\alpha_v\beta_3$ and - $\alpha_v\beta_5$ monoclonal antibodies to that observed on cells adherent to intact vitronectin (Fig. 2B).

To further characterize the ability of the heparin-binding region of vitronectin to inhibit the cell surface expression of matrix assembly sites, intact vitronectin was preincubated with increasing concentrations of heparin prior to coating onto tissue culture wells. Following a 3-h incubation, unbound protein was removed, and wells were washed with PBS prior to the seeding of cycloheximide pretreated fibroblasts. As demonstrated in Fig. 3, pretreatment of vitronectin with heparin prevented the inhibitory effect of vitronectin on ¹²⁵I-labeled

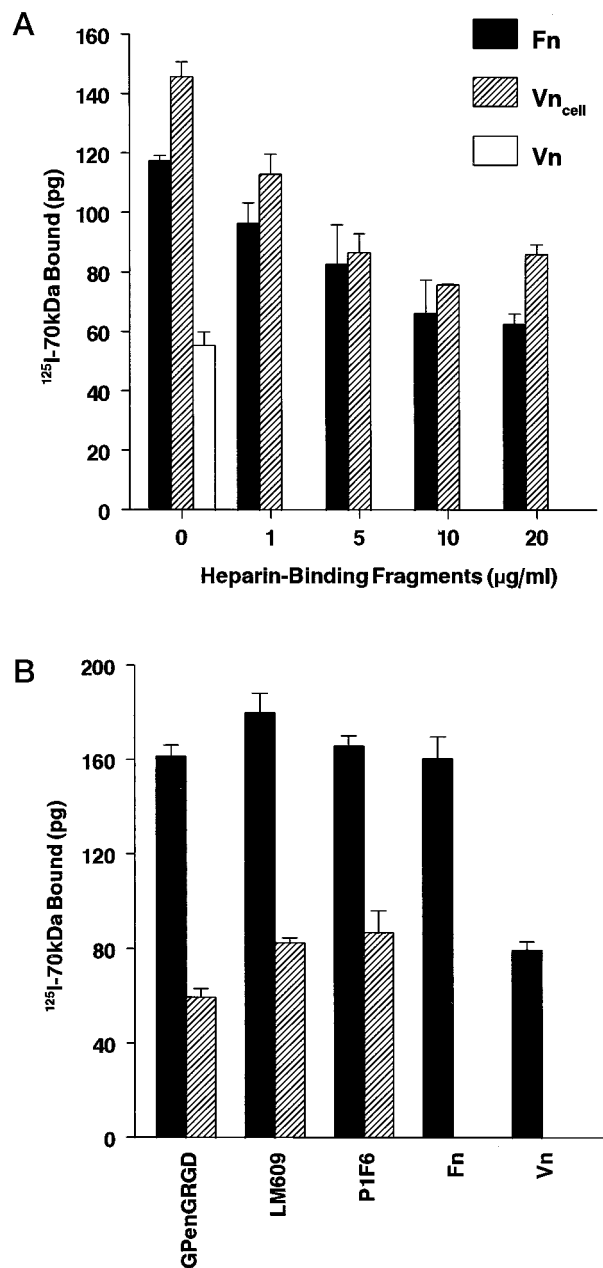


FIG. 2. Effect of heparin-binding fragments of vitronectin on ¹²⁵I-labeled 70-kDa fragment binding. A, cycloheximide-pretreated fibroblasts were seeded onto tissue culture wells precoated with 10 μ g/ml of the 40-kDa cell-binding fragment of vitronectin (hatched bars) or intact fibronectin (solid bars) in the absence (0) or presence of increasing concentrations of heparin-binding fragments of vitronectin. Cells were also seeded onto intact vitronectin (open bars). B, cells were seeded onto either vitronectin integrin receptor-specific peptides (GPenGRGD), anti-integrin $\alpha_v\beta_3$ (LM609) or $\alpha_v\beta_5$ (P1F6) antibodies in the absence (solid bars) or presence (hatched bars) of 20 μ g/ml heparin-binding fragments of vitronectin. Cells were also seeded onto intact fibronectin (Fn) or intact vitronectin (Vn). The ¹²⁵I-labeled 70-kDa fragment binding assay was performed as indicated in the legend to Fig. 1. Data are presented as ¹²⁵I-labeled 70-kDa fragment bound per well \pm range (A) or S.E. (B).

70-kDa fragment binding and resulted in a dose-dependent restoration of ¹²⁵I-labeled 70-kDa fragment binding to levels observed on fibronectin-adherent cells. Taken together, these data indicate that ligation of either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins with the RGD sequence of vitronectin is permissive for matrix assembly site expression, while the interaction of cells with a region in or near the heparin-binding domain of vitronectin

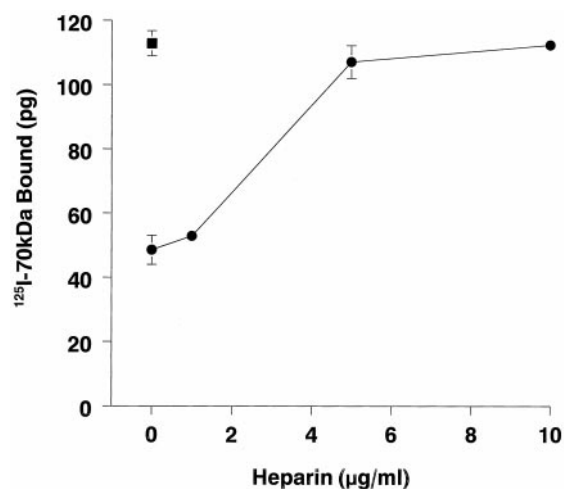


FIG. 3. **Expression of 70-kDa binding sites on cells adherent to heparin-treated vitronectin.** Tissue culture wells were coated with 10 µg/ml untreated fibronectin (■) or vitronectin preincubated for 30 min with increasing concentrations of heparin (●). Wells were washed with PBS, and cycloheximide-pretreated fibroblasts were seeded at 10^5 in DMEM/ITS+2 with 20 µg/ml cycloheximide and allowed to adhere and spread for 18 h. The ^{125}I -labeled 70-kDa fragment binding assay was performed as indicated in the legend to Fig. 1. Data are presented as the amount of ^{125}I -labeled 70-kDa fragment bound per well \pm S.E.

leads to a down-regulation of cell surface matrix assembly site expression.

Localization of the Inhibitory Effect of Vitronectin on Matrix Assembly Site Expression to the Heparin-binding Domain—To more precisely localize the region of vitronectin responsible for the inhibition of matrix assembly site expression, a recombinant GST fusion protein containing a 39-amino acid sequence encompassing the heparin-binding domain of vitronectin (32, 51) was constructed and tested for its ability to alter ^{125}I -labeled 70-kDa fragment binding. As shown in Fig. 4, the addition of increasing concentrations of the heparin-binding domain of vitronectin to cells seeded onto fibronectin-coated dishes resulted in a dose-dependent decrease in ^{125}I -labeled 70-kDa fragment binding. This inhibitory effect was maximal at 0.9 µM Vn_{HBD} and was specific to the heparin-binding domain of vitronectin. The addition of equal molar concentrations of either GST alone or the heparin-binding domain of fibronectin (GST/FNIII_{12,13}) (34, 54) to fibronectin-adherent cells had no effect on ^{125}I -labeled 70-kDa fragment binding (Fig. 4). In addition, incubation of fibronectin-adherent cells with either intact recombinant vitronectin or a recombinant vitronectin in which the RGD sequence had been mutated to RGE ($r\text{Vn}_{\text{RGE}}$) resulted in inhibition of ^{125}I -labeled 70-kDa fragment binding (Fig. 4). This inhibitory effect was maximal at 0.35 µM, providing further evidence that the inhibitory effect of vitronectin on matrix assembly site expression is not due to integrin ligation via the RGD sequence of vitronectin. Taken together, these data indicate that the interaction of cells with the heparin-binding domain of vitronectin leads to a down-regulation of matrix assembly site expression.

Vitronectin's Heparin-binding Domain Inhibits Fibronectin Polymerization—Upon binding of the amino-terminal region of fibronectin to the cell surface, fibronectin accumulates in the extracellular matrix in the form of disulfide-stabilized aggregates (45). As such, the ability of vitronectin's heparin-binding domain to down-regulate matrix assembly site expression indicates a role for the heparin-binding domain of vitronectin in the regulation of fibronectin polymerization. To determine whether the interaction of cells with the heparin-binding domain of vitronectin inhibits endogenous fibronectin matrix as-

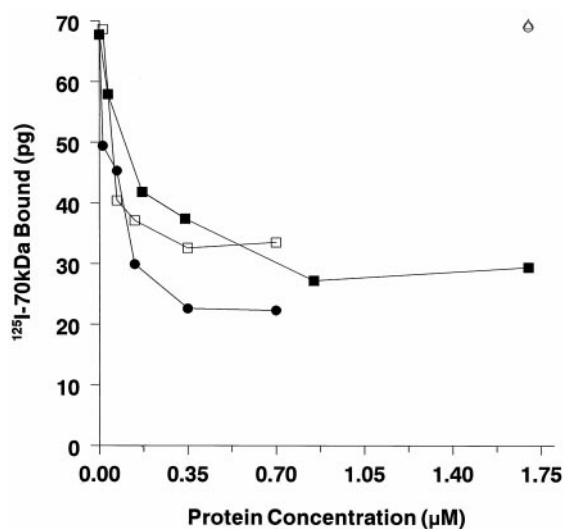


FIG. 4. **Effect of the heparin-binding domain of vitronectin on ^{125}I -labeled 70-kDa fragment binding.** Cycloheximide-pretreated fibroblasts were seeded onto fibronectin-coated tissue culture wells and allowed to adhere and spread for 45 min prior to the addition of GST/ Vn_{HBD} (■), recombinant vitronectin (●), recombinant Vn_{RGE} (□), GST/FNIII_{12,13} (○), or GST (△). Cells were incubated an additional 15 h at 37 °C. The ^{125}I -labeled 70-kDa fragment binding assay was performed as indicated in the legend to Fig. 1. Data are presented as the amount of ^{125}I -labeled 70-kDa fragment bound per well \pm S.E.

sembly, non-cycloheximide-treated fibroblasts were seeded onto fibronectin-coated coverslips in the absence or presence of either the heparin-binding domain of vitronectin (1.7 µM), the heparin-binding domain of fibronectin (1.7 µM), or intact recombinant vitronectin (0.75 µM). These concentrations were chosen to ensure maximum inhibition of 70-kDa fragment binding (see Fig. 4). As demonstrated in Fig. 5A, control fibroblasts elaborated an extensive fibrillar fibronectin matrix. Similar fibrillar fibronectin staining was observed on cells that had been incubated in the presence of GST/FNIII_{12,13} (Fig. 5B). In contrast, treatment of cells with either the heparin-binding domain of vitronectin (Fig. 5C) or intact recombinant vitronectin (Fig. 5D) markedly reduced the assembly of a fibronectin matrix. These data demonstrate that the interaction of cells with the heparin-binding domain of vitronectin inhibits endogenous fibronectin fibril formation and suggests a role for vitronectin in the regulation of fibronectin matrix assembly.

Inhibition of Matrix Assembly Site Expression on β_1 Null Cells—Several recent studies indicate that fibronectin-binding integrins other than $\alpha_5\beta_1$, including $\alpha_3\beta_1$ (55), "activated" $\alpha_v\beta_3$ (56), and "activated" $\alpha_{\text{Iib}}\beta_3$ (22), can support fibronectin matrix assembly. To determine whether the heparin-binding domain of vitronectin also down-regulates non- β_1 integrin-mediated matrix assembly site expression, cycloheximide-pretreated β_1 -null (GD25) and β_1 -transfected (GD25 β_1) cells were seeded onto fibronectin-coated tissue culture wells in the absence or presence of either intact vitronectin or the heparin-binding domain of vitronectin. These cells, which lack β_1 -containing integrin receptors due to a targeted knockout of the β_1 gene (57), have been shown to assemble a fibronectin matrix through a mechanism that is thought to be dependent on the $\alpha_v\beta_3$ integrin (29). As shown in Fig. 6A, the addition of vitronectin to either A1F fibroblasts, β_1 -null, or β_1 -transfected cells resulted in a similar inhibition of ^{125}I -labeled 70-kDa fragment binding. Similar to the results obtained using A1F fibroblasts (Figs. 1B and 4), the inhibitory effect of vitronectin on ^{125}I -labeled 70-kDa fragment binding to GD25 and GD25 β_1 cells was mediated by the heparin-binding domain of vitronectin and was reversed by treatment of cells with LPA (Fig. 6B). These studies indicate

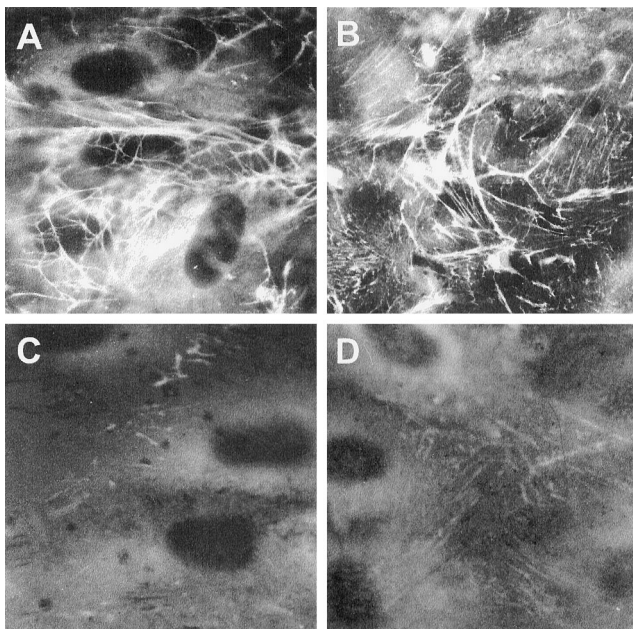


FIG. 5. Effect of the heparin-binding domain of vitronectin on endogenous fibronectin matrix assembly. Fibroblasts (10^5 in DMEM/TTS+2) were seeded onto coverslips precoated with $5 \mu\text{g/ml}$ of fibronectin and allowed to adhere and spread for 45 min prior to the addition of either PBS (A), GST/FnIII_{12,13} (B), GST/Vn_{HBD} (C), or recombinant vitronectin (D). Cells were incubated an additional 15 h at 37°C . Cells were processed for immunofluorescence as indicated under "Experimental Procedures." Fibronectin fibrils were visualized using a polyclonal anti-fibronectin antibody followed by a fluorescein isothiocyanate-labeled goat anti-rabbit antibody.

that the interaction of cells with the heparin-binding domain of vitronectin down-regulates both β_1 and non- β_1 integrin-mediated matrix assembly site expression.

The Heparin-binding Domain of Vitronectin Induces Actin Filament Reorganization—Several studies have correlated changes in the organization of the actin cytoskeleton with changes in 70-kDa binding site expression and fibronectin matrix deposition (18, 21–23). To determine whether the heparin-binding domain of vitronectin induced changes in the distribution of actin filaments, serum-starved, non-cycloheximide-treated fibroblasts were seeded onto fibronectin-coated coverslips in the absence or presence of the heparin-binding domains of either vitronectin or fibronectin. Cells were allowed to adhere and spread overnight, and actin was visualized by staining fixed and permeabilized cells with fluorescein isothiocyanate-conjugated phalloidin. As shown in Fig. 7, in both GST-treated cells (panel A) and GST/FnIII_{12,13}-treated cells (panel E), actin filaments were organized into thickly bundled stress fibers commonly observed in fibronectin-adherent cells. In contrast, treatment of cells with the heparin-binding domain of vitronectin ($3.4 \mu\text{M}$) resulted in the organization of actin into loose, broadly distributed microfilament nets (Fig. 7C). Under these conditions, 100% of the cells exhibited changes in actin organization. At lower concentrations of Vn_{HBD} ($1.7 \mu\text{M}$), approximately 50% of the cells exhibited readily visible changes in actin microfilament organization (data not shown). Vinculin, a protein that is highly concentrated at focal adhesions (58), was clearly visible under all experimental conditions (Fig. 7, B, D, and F).

Modulation of Cytoskeletal Interactions by the Heparin-binding Domain of Vitronectin—To further characterize the vitronectin-induced changes in actin cytoskeletal organization, the subcellular distributions of actin-associated proteins from control and GST/Vn_{HBD}-treated cells were examined. As demonstrated in Fig. 8, treatment of cells with the heparin-binding

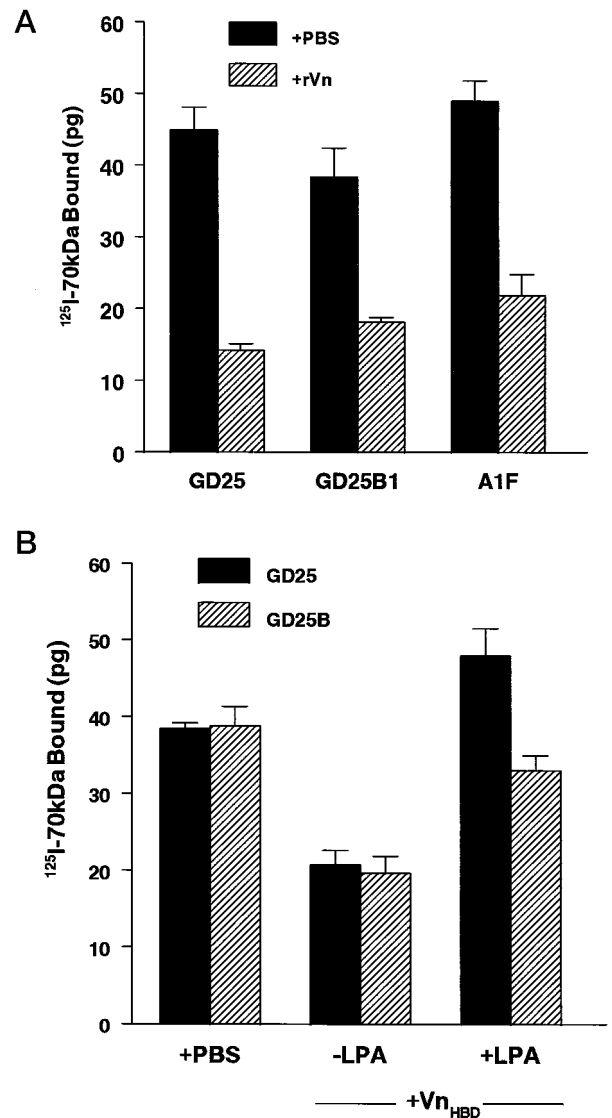


FIG. 6. Effect of vitronectin on ^{125}I -labeled 70-kDa fragment binding to β_1 -null cells. A, cycloheximide-pretreated A1F fibroblasts, GD25, or GD25 β_1 cells were seeded at confluence onto fibronectin-coated tissue culture wells and allowed to adhere and spread for 45 min prior to the addition of either recombinant Vn (hatched bars) or an equal volume of PBS (solid bars). The ^{125}I -labeled 70-kDa fragment binding assay was performed as indicated in the legend to Fig. 1. B, GD25 (solid bars) or GD25 β_1 cells (hatched bars) were seeded onto fibronectin-coated wells and treated with either the heparin-binding domain of vitronectin (+Vn_{HBD}) or PBS. One hour prior to the assay, some cells treated with GST/VnHBD were incubated with $2 \mu\text{M}$ LPA (+LPA). Data are presented as ^{125}I -labeled 70-kDa fragment bound per well \pm S.E.

domain of vitronectin resulted in a significant decrease in the level of α -actinin associated with the digitonin-soluble pool as compared with levels observed in either control or GST/FnIII_{12,13}-treated cells. This decrease in soluble α -actinin was accompanied by an increase in the level of α -actinin associated with the digitonin-insoluble fraction (Fig. 8), suggesting that stimulation of cells with the heparin-binding domain of vitronectin triggers the redistribution of soluble α -actinin to the actin cytoskeleton. To examine the distribution of other actin-associated proteins, immunoblots were stripped and sequentially reprobed with antibodies directed against either talin or vinculin (58). In contrast with the results obtained with α -actinin (Fig. 8), treatment of cells with the heparin-binding domain of vitronectin resulted in an increase in the level of

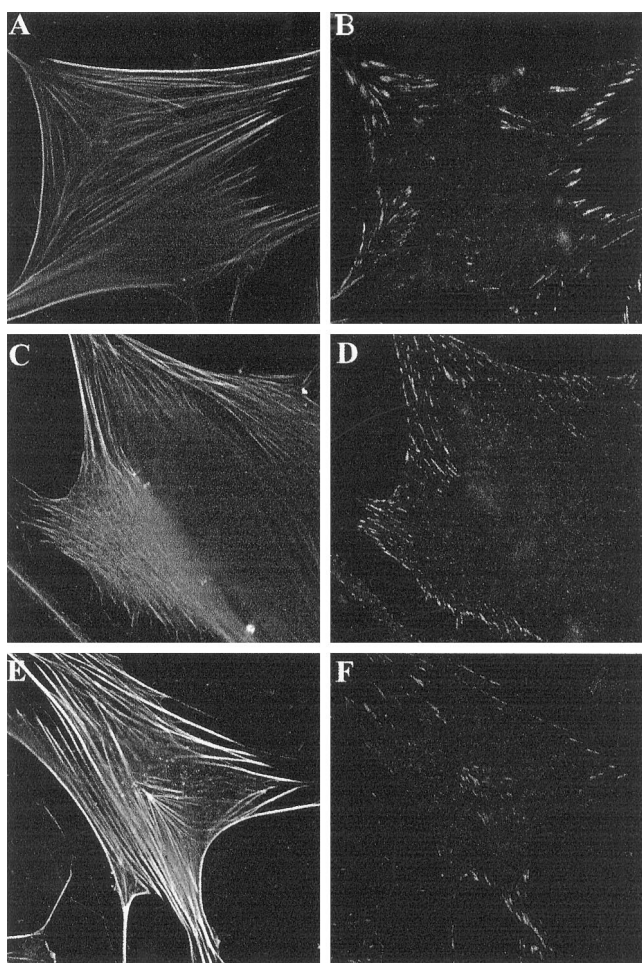


FIG. 7. Effect of vitronectin's heparin-binding domain on actin organization. Serum-starved, non-cycloheximide-treated fibroblasts were resuspended in DMEM/ITS+2 at 2.5×10^4 cell/ml and seeded onto fibronectin-coated coverslips in the presence of $3.4 \mu\text{M}$ of GST (A and B), GST/Vn_{HBD} (C and D), or GST/FnIII_{12,13} (E and F). Following an 18-h incubation, cells were fixed and permeabilized, and actin filaments were visualized by staining with fluorescein isothiocyanate-phalloidin (A, C, and E). Cells were co-stained for vinculin using an anti-vinculin monoclonal antibody followed by a Texas Red-labeled goat anti-mouse antibody (B, D, and F). Results are representative of three separate experiments.

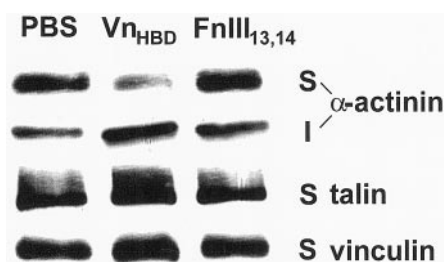


FIG. 8. Subcellular distribution of actin-associated proteins. Serum-starved, non-cycloheximide-treated fibroblasts were resuspended in DMEM/ITS+2 and seeded onto fibronectin-coated coverslips in the presence of $3.4 \mu\text{M}$ of GST/Vn_{HBD} (Vn_{HBD}), GST/FnIII_{12,13} (FnIII_{12,13}), or an equal volume of PBS (PBS). Following an 18-h incubation, cells were washed and extracted sequentially with digitonin (S) and radioimmune precipitation (I) buffers. Fractions (10 μg /lane) were resolved by electrophoresis and analyzed by immunoblotting with monoclonal antibody specific for α -actinin. The blot was then stripped and sequentially reprobed with monoclonal antibodies specific for talin and vinculin. Results are representative of three separate experiments.

talin detected in the soluble fraction when compared with either control or GST/FnIII_{12,13}-treated cells (Fig. 8). No significant differences in the levels of vinculin staining were observed

in the soluble fractions obtained from either control or GST/FnIII_{12,13}- or GST/Vn_{HBD}-treated cells (Fig. 8). Taken together, these data indicate that the interaction of fibronectin-adherent cells with the heparin-binding domain of vitronectin stimulates the organization of actin into microfilament nets concomitant with the subcellular reorganization of specific actin-associated proteins, including α -actinin and talin.

DISCUSSION

In the present study, we demonstrate that the interaction of cells with the heparin-binding region of vitronectin inhibits fibronectin matrix assembly site expression and fibronectin deposition. The inhibitory effect of vitronectin's heparin binding domain was seen in both β_1 -dependent and β_1 -independent matrix assembly and could be overcome by treatment of cells with LPA, an agent known to increase actin stress fiber formation (18, 24). Moreover, the interaction of fibronectin-adherent cells with the heparin-binding domain of vitronectin resulted in changes in the cytoskeletal organization of actin, as well as the actin-binding proteins α -actinin and talin.

Several previous studies have demonstrated that modulation of the actin cytoskeleton alters the ability of cells to assemble a fibronectin matrix (18, 21–24). Our data indicate that inhibition of fibronectin matrix assembly by the heparin-binding domain of vitronectin is associated with the formation of microfilamentous nets of actin and a marked redistribution of the actin binding protein α -actinin to the cytoskeleton associated pool. These cytoskeletal changes were specific for the heparin-binding domain of vitronectin, since differences were not observed upon treatment of cells with the heparin-binding domain of fibronectin. Our findings are in agreement with the recent study by Zhang *et al.* (27), which shows that the down-regulation of matrix assembly by cells adherent to vitronectin is accompanied by changes in cell morphology. Previous studies have demonstrated agonist-induced redistribution of cytoskeletal and signaling proteins in platelets (59, 60) and neutrophils (61). Thus, it is possible that the redistribution of cytoskeletal components upon treatment of cells with the heparin-binding domain of vitronectin serves to localize or sequester signaling enzymes involved in regulating matrix assembly.

Recently, it has been shown that LPA up-regulates expression of matrix assembly sites as well as fibronectin polymerization through the Rho-dependent activation of cellular contraction (23, 24). This finding suggests that one role for organized actin in matrix assembly is to generate the contractile forces necessary for fibronectin polymerization. Earlier studies have shown that in the absence of β_1 integrins, β_3 integrins can regulate matrix assembly (22, 29, 56). Vitronectin inhibited matrix assembly site expression on β_1 null cells, suggesting that vitronectin is able to modulate fibronectin matrix assembly regulated by either β_3 or β_1 integrins. The ability of LPA to overcome vitronectin inhibition of matrix assembly suggests that the inhibitory signal from vitronectin may be modulating a pathway important in maintaining levels of contractility sufficient to promote matrix assembly site expression. In contrast, treatment of vitronectin-adherent cells with PMA, an agent that does not increase fibroblast contractility (62), did not result in an increase in matrix assembly site expression. Recent studies have shown that vitronectin-mediated down-regulation of 70-kDa binding sites can be partially reversed by the expression of the β_1 integrin and completely reversed when cells expressing β_1 are treated with LPA (27). Therefore, the results from our study as well as the study by Zhang *et al.* (27) are consistent with a mechanism in which maximum expression of fibronectin matrix assembly sites requires both β_1 integrin and appropriate levels of cellular contractility.

The effect of vitronectin's heparin-binding domain on fi-

bronectin matrix assembly may be mediated by the $\alpha_v\beta_5$ integrin or by heparan sulfate proteoglycans. Vogel *et al.* (63) have demonstrated by affinity chromatography that the $\alpha_v\beta_5$ integrin receptor binds to a peptide derived from the heparin-binding domain of vitronectin. This non-RGD-containing peptide is composed of 12 amino acids that are also contained within the GST/Vn_{HBD} construct. Vitronectin has also been shown to colocalize with heparan sulfate proteoglycans on endothelial cells (64). Removal of cell surface proteoglycans by treatment with heparinase (36), β -D-xylosidase (36), or sodium chlorate (65) inhibits the binding of multimeric vitronectin to cell surfaces, suggesting that the binding of vitronectin to cells is mediated by an interaction between heparan sulfate proteoglycans and the heparin-binding domain of vitronectin.

Extrahepatic synthesis of vitronectin has been demonstrated to be spatially and temporally regulated during development and progression of certain tumors. Vitronectin synthesis is increased in association with migrating cells (66, 67) and in several tumors (68–70). Our data support the hypothesis that, *in vivo*, vitronectin may serve as a physiologic ligand responsible for the down-regulation of fibronectin matrix assembly sites on fibroblasts at the tumor-stromal interface. Local synthesis of vitronectin would be expected to signal a decrease in the levels of matrix assembly site expression by stromal fibroblasts. Since previous studies have demonstrated that decreased cell migration rates are associated with increased levels of polymerized fibronectin (7), dampening of matrix assembly pathways by vitronectin would provide a mechanism to facilitate tumor invasion of local connective tissue. The synthesis and deposition of vitronectin into the stroma of colorectal adenocarcinoma by stromal fibroblasts (69) may reflect an autocrine mechanism that promotes fibroblast remodeling of the stromal matrix. Recent studies have proposed that maintenance of cellular and tissue architecture may repress the tumor phenotype (71). The role of vitronectin in promoting the matrix remodeling that contributes to the disruption of local tissue architecture associated with tumor progression remains an important avenue of future investigation.

Acknowledgments—We are grateful to Renotta Smith, Eiman Sebald, and David Wagoner for providing technical assistance and to Dr. Susan LaFlamme (Albany Medical College) for critically reviewing this manuscript.

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Inhibition of Fibronectin Matrix Assembly by the Heparin-binding Domain of Vitronectin

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J. Biol. Chem. 1999, 274:27257-27264.
doi: 10.1074/jbc.274.38.27257

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