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A Novel Peptide Sequence in Perlecan Domain IV Supports Cell Adhesion, Spreading and FAK Activation

Mary C. Farach-Carson^{*}, Anissa J. Brown, Megan Lynam, Jeffrey B. Safran, and Daniel D. Carson

Department of Biological Sciences, University of Delaware, Newark, DE, 19716 USA

Abstract

Perlecan/HSPG2 is a large, multi-domain, multifunctional heparan sulfate proteoglycan with a wide tissue distribution. With the exception of its unique domain I, each of perlecan's other four domains shares sequence similarity to other protein families including low density lipoprotein (LDL) receptor, laminin alpha chain, neural cell adhesion molecule (NCAM), immunoglobulin superfamily members, and epidermal growth factor (EGF). Previous studies demonstrated that glycosaminoglycan-bearing perlecan domain I supports early chondrogenesis and growth factor delivery. Other sites in the core protein interact with other matrix molecules and support cell adhesion, although the peptide sequences involved remain unidentified. To identify novel functional motifs within perlecan, we used a bioinformatics approach to predict regions likely to be on the exterior of the folded protein. Unique hydrophilic sequences of about 18 amino acids were selected for testing in cell adhesion assays. A novel peptide sequence (TWSKVGGHLRPGIVQSG) from an immunoglobulin (Ig) repeat in domain IV supported rapid cell adhesion, spreading and focal adhesion kinase (FAK) activation when compared to other peptides, a randomly scrambled sequence of the domain IV peptide or a negative control protein. MG-63 human osteosarcoma cells, epithelial cells and multipotent C₃H10T1/2 cells, but not bone marrow cells, rapidly, i.e., within 30 min, formed focal adhesions and assembled an actin cytoskeleton on domain IV peptide. Cell lines differentially adhered to the domain IV peptide, suggesting adhesion is receptor specific. Adhesion was divalent cation independent and heparin sensitive, a finding that may explain some previously poorly understood observations obtained with intact perlecan. Collectively, these studies demonstrate the feasibility of using bioinformatics-based strategies to identify novel functional motifs in matrix proteins such as perlecan.

Keywords

perlecan; immunoglobulin repeat; cell adhesion; extracellular matrix; heparan sulfate proteoglycan; peptide

Introduction

Perlecan (HSPG2) is a large heparan sulfate proteoglycan expressed in a wide variety of tissues and tissue structures including cartilage and basal lamina (Hassell, J. et al., 2002). Perlecan has been identified in species ranging from insects and nematodes to mammals (Hassell, J. et

^{*}Corresponding Author: Department of Biological Sciences, University of Delaware, 326 Wolf Hall, Newark, DE 19716; Phone: 302-831-2277; Fax: 302-831-2281; Email: farachca@udel.edu.

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al., 2002; Rogalski, T.M. et al., 2001; Voigt, A. et al., 2002). Severe developmental defects are associated with perlecan mutations in all species examined to date (Arikawa-Hirasawa, E. et al., 1999; Arikawa-Hirasawa, E. et al., 2001; Costell, M. et al., 1999; Nicole, S. et al., 2000; Rogalski, T.M. et al., 2001; Voigt, A. et al., 2002). Perlecan participates in diverse biological functions including growth factor delivery, promotion of cell adhesion, proliferation and cancer progression (Farach-Carson, M.C. et al., 2005; Iozzo, R.V., 2005; Savore, C. et al., 2005; Yang, W.D. et al., 2005). This spectrum of functions is reflected by the complexity of the protein core consisting of five distinct, independently functioning domains (figure 1). Unique domain I contains three glycosaminoglycan attachment sites as well as an SEA domain (Dolan, M. et al., 1997). The other four domains possess sequence similarity to other protein families (Murdoch, A.D. et al., 1992; Noonan, D.M. et al., 1991, Farach-Carson and Carson, 2007). Domain II contains repeat sequences highly similar to LDL-receptor, while domain III is comprised of tandem globular domain repeats similar to domain IV of the laminin alpha (A) chain and containing both laminin EGF-like and laminin G (LG) like repeats. An RGD motif is found in mouse, but not human, perlecan domain III (Chakravarti et al, 1995). Domain IV contains repeats similar to those found in the immunoglobulin (Ig) superfamily member, neural cell adhesion molecule (N-CAM) or platelet endothelial cell adhesion molecule (PECAM/CD31). The C-terminal domain V shows sequence similarity to G region of the laminin alpha (A) chain. There also are epidermal growth factor (EGF)-like sequences spaced between the domain G-like repeats in perlecan domain V. Some studies indicate that glycosaminoglycan modification can occur on domain V (Friedrich, M.V. et al., 1999; Tapanadechopone, P. et al., 1999).

Each domain of perlecan previously has been produced as a recombinant protein and functional studies have been performed demonstrating that each domain can function independently. In particular, domain IV has been shown to support a variety of activities including promotion of cell adhesion and interaction with platelet derived growth factor, heparin/sulfatides, nidogens, fibronectin and fibulin-2 (Hopf, M. et al., 1999; Hopf, M. et al., 2001). Approaches to dissecting the functions of various perlecan domains have relied almost entirely upon expression of recombinant fragments of the protein or generation of various mutations followed by functional studies. The improved performance and application of protein structure-predicting algorithms provides new opportunities to identify motifs in complex proteins likely to interact with other proteins and directly participate in biological processes. In the present study, we have utilized a bioinformatics and structural modeling approach to predict peptide motifs within each perlecan domain that are unique, most likely to be exposed on the surface of the protein and, therefore, available to interact with other proteins and cells. As a result, a novel surface disposed motif in the Ig14 (mouse)/Ig21 (human) loop of domain IV was identified that uniquely supports cell adhesion and spreading of specific cell lines including both mesenchymal (fibroblasts and osteoblasts) and epithelial cells.

Results

Prediction of active peptide sequences

As mentioned above, perlecan is composed of five domains (mouse protein shown in figure 1). Hydrophilicity profiles were constructed for each of these domains to predict which regions and peptide motifs were most likely to be exposed on the surface of these domains where they could interact with other proteins and cell surfaces (only shown for the domain IV Ig repeat in figure 2A, also shown in classic two dimensional representation in figure 2B). As a result, five peptide sequences within perlecan were chosen based on their predicted exposure at the protein surface, conservation between human and mouse, and lack of sequence identity to other peptide sequences with known adhesive activity. Three dimensional homology modeling also was performed for domain IV as described in "Experimental Procedures". The model shown in

figure 3 was generated using 3D-PSSM and its successor Phyre, reporting a 100% estimated precision value for an Ig I-Set conformation with the characteristic core Greek-key beta-sandwich structure. The peptide sequence that we identified (perlecan domain IV peptide) is predicted to lie on an externally disposed “finger” protruding from the beta barrel (arrow).

Cell adhesion to peptides

We next sought to determine if any of the peptides we identified in this fashion had cell adhesive activity (figure 4). Each candidate peptide identified by the above analyses was tested for its ability to directly support cell attachment using a direct coupling procedure in which the peptides were covalently linked directly to derivatized nonadhesive plastic surfaces. As shown in figure 4, cells adhered somewhat to peptides I and II, derived from the similar LDL-receptor class A motifs. They also adhered comparably to peptide V, the laminin G-like derived sequence. Cells bound very poorly, if at all, to peptide III, derived from the laminin EGF-like module. In contrast, cells adhered well to the peptide IV derived from the Ig repeat in domain IV. Adhesion to this peptide at 1 µg/ml was higher than to any other peptide we tested, even at a ten-fold higher concentration of the other peptides. Because of this adhesion-promoting activity, we chose to use this peptide for more detailed studies. Because of the relatively low level of binding owed to low surface density of the pure peptide, we subsequently used a second assay based on our previous work examining cell adhesion to perlecan. Using standard tissue culture plates, non-specific binding sites can be blocked with heat-denatured BSA prior to performing cell adhesion assays using C₃H10T1/2 cells, a cell line previously shown to bind and spread on the surfaces coated with recombinant fragments of all five perlecan domains in the absence of glycosaminoglycan (French, M.M. et al., 2002; French, M.M. et al., 1999), or using other cell types. BSA blocked plates thus served as the negative control in all assays. Type I collagen was used as the positive control.

For convenience and to examine the functionality of perlecan domain IV peptide in a multivalent configuration, we covalently conjugated this peptide to BSA. As shown in figure 5, the conjugation process generated a range of conjugate masses from approximately 68,200 to 92,000 with a median molecular mass of 79,535. Based on the molecular masses determined for the peptide and BSA starting material, it could be calculated that the number of peptides conjugated to BSA in these preparations ranged from 1 to 14 with a median of approximately 7 peptides/BSA molecule. These preparations were used in further experiments.

BSA-conjugated perlecan domain IV peptide was used at various concentrations to coat plastic surfaces to test in cell adhesion assays. MG-63 human osteosarcoma cells (Booyens, J. et al., 1984) and C₃H10T1/2 pluripotential fibroblasts were used in these and additional studies with similar results. Figure 6 shows that MG-63 cells bound to perlecan domain IV peptide-BSA conjugate coated surfaces in a peptide dose-dependent fashion. At coating concentrations above 0.1 mg/ml, adhesion was comparable to that obtained using type I collagen which was higher than that on tissue culture plastic alone (PBS control). In contrast, adhesion to BSA-coated surfaces was almost undetectable. A randomly scrambled sequence of perlecan domain IV peptide (Scr Pln IV in figure) was used as a control for sequence specificity and showed very little ability to support cell attachment even when used to coat surfaces at concentrations up to 1 mg/ml. It was concluded that perlecan domain IV peptide provided a useful support for cell attachment and that the peptide sequence, rather than the simple composition of the peptide or the BSA carrier, was critical for this activity.

As an additional test of the specificity of the perlecan domain IV peptide in supporting cell attachment, we examined the ability of several more human cell lines to attach to BSA conjugates made from perlecan domain IV peptide or the scrambled perlecan peptide IV sequence. In addition to MG63 cells, two human epithelial cell lines, the non-transformed uterine epithelial cell line, HES (Desai, N.N. et al., 1994), and the prostate carcinoma cell line,

PC3 (Weber et al., 1996), bound well to perlecan domain IV peptide, but not its scrambled counterpart. In contrast, two cell lines derived from bone marrow stroma, HS27a and HS5 (Roeklein, B.A. and Torok-Storb, B., 1995) failed to bind to either sequence although both adhered well to collagen (data not shown). It was concluded that cell attachment to perlecan domain IV peptide displayed cell line specificity, presumably due to differences in expression of complementary binding proteins or receptors on the surfaces of these cell lines.

Binding to perlecan domain IV peptide organizes the actin cytoskeleton

The cell attachment assays routinely used in the above studies did not discriminate between simple binding *versus* binding and spreading. The latter events are energy dependent and generally are associated with interactions with cell surface receptors, activation of signal transduction cascades and cytoskeletal reorganization (Gumbiner, B.M., 1996). Consequently, we used light and confocal microscopy to detect gross morphological evidence of cell spreading as well as staining with fluorescently-labeled phalloidin to detect the formation and organization of filamentous actin in MG-63 cells on perlecan domain IV peptide coated surfaces. As shown in figure 8, the few cells that bound to BSA or perlecan scrambled peptide IV coated surfaces did not display either obvious spreading or significant binding of the phalloidin probe indicating a lack of filamentous actin formation. In contrast, both cell spreading and robust binding of phalloidin was observed on surfaces coated with either type I collagen or perlecan domain IV peptide. Furthermore, staining was particularly robust at the cell peripheries in both cases, consistent with active spreading on these surfaces. Experiments performed with C₃H10T1/2 cells also indicated that binding to perlecan domain IV peptide was accompanied by increased focal adhesion kinase (FAK) phosphorylation on tyrosine 397 (figure 9). Similar FAK activation was seen with MG-63 cells (data not shown). It was concluded that cell binding to perlecan domain IV peptide was accompanied by spreading, cytoskeletal organization, and FAK activation, all activities expected for receptor-mediated cell adhesion processes.

Characteristics of cell surface perlecan domain IV peptide binding proteins

As an initial approach to characterize the cell surface sites involved in binding to perlecan peptide IV, we utilized an antibody blocking approach with a function-blocking anti-human β 1 integrin, chosen for its broad applicability (Matlin, K.S. et al., 2003). Using type I collagen, a well-established integrin β 1 ligand (Heino, J., 2000) as a control, it was determined that a 60 min preincubation of MG63 cells was required for robust (80–90%) inhibition of β 1 integrin-dependent binding. Under these conditions, anti- β 1 inhibited binding to perlecan peptide IV no more than 50%, with large error bars (data not shown). Likewise, cell attachment was not influenced by the presence of divalent cations: neither Ca^{2+} nor Mg^{2+} affected binding (figure 10), indicating that, if integrins are involved, the interactions are not of a conventional Ca^{2+} dependent type and they are likely to occur indirectly via receptor cross talk with other surface binding proteins.

We next tested if binding of C₃H10T1/2 cells to perlecan domain IV peptide BSA conjugate was inhibited by the inclusion of the glycosaminoglycans heparin or chondroitin sulfate. As shown in figure 11, chondroitin sulfate had no effect on binding of cells when used at concentrations up to 200 $\mu\text{g/ml}$. In contrast, 20 $\mu\text{g/ml}$ heparin markedly inhibited (>95%) binding of the cells to perlecan domain IV peptide. This dramatic finding is consistent with earlier findings in our laboratory demonstrating that this cell line also does not bind to intact perlecan or perlecan domain I when decorated with heparan sulfate, but binds readily when the heparan sulfate is removed enzymatically.

Discussion

In previous studies, we reported the ability of perlecan to initiate aggregate formation and chondrocytic differentiation of pluripotent C₃H10T1/2 cells (French, M.M. et al., 1999). This phenomenon involving cell-cell interactions occurs when dishes are coated with the intact perlecan molecule or with recombinant glycosaminoglycan-bearing perlecan domain I (French, M.M. et al., 2002), but not when the heparan sulfate is removed. In this case, cells attach and spread on all domains to form smooth monolayers. The most obvious explanation for this phenomenon is that perlecan contains adhesive motifs that are made cryptic by the presence of the large heparan sulfate chains. To identify these novel bioactive motifs in the large multifunctional perlecan molecule, polypeptides representing distinct structural domains were designed and tested for the ability to support cell adhesion. After culturing the cells on peptides from each domain individually, it was found that the domain IV peptide from perlecan uniquely supported adhesion of C₃H10T1/2 cells used in the initial screen. No other domain peptide had this clear activity, and C₃H10T1/2 cells rapidly attached and spread on perlecan domain IV peptide. This indicated that of the five candidate peptides identified by structural analysis, only one was able to present cells with a viable motif for recognition by surface receptors. Additional studies examining the adhesive behavior of a variety of human cell lines demonstrated that adhesion to the peptide motif identified in domain IV was cell line as well as sequence specific because a randomly scrambled version of the peptide sequence failed to support cell attachment in any context.

We previously suggested that C₃H10T1/2 cells when cultured on intact perlecan or domain I failed to form stable focal adhesions with the culture substratum, and thus they rounded up, aggregated, and entered an early chondrogenesis program (French, M.M. et al., 2002). Other domains of perlecan, or perlecan domain I lacking glycosaminoglycan chains, are devoid of this activity and permit cells to form focal adhesions and continue to proliferate without signs of differentiation. The adhesion of cells to other perlecan domains or to perlecan lacking glycosaminoglycan chains pointed to the presence of cell adhesive domains within the perlecan core structure that are only functional when separated from the dominant anti-adhesive activity of fully glycosylated perlecan domain I. Such molecular separation could readily occur during proteolytic processing or catabolism of perlecan. Proteolytic processing of perlecan occurs *in vivo* in certain tissues (Balasubramani, M. et al., 2004; Laplante, P. et al., 2006; Oda, O. et al., 1996; Raymond, M.A. et al., 2004; Thadikaran, L. et al., 2005; Farach-Carson and Carson, 2007). Moreover, several extracellular proteases including plasmin, MMP3 (stromelysin) and MMP13 (collagenase-3) have been shown to hydrolyze perlecan in a fashion that would separate glycosaminoglycan-bearing domain I from other portions of perlecan (Whitelock, J.M. et al., 1996). Thus, it is likely that extracellular processing occurs *in vivo* in a fashion that not only can influence growth factor retention in the extracellular matrix, but also modulate adhesive and anti-apoptotic functions of perlecan (Laplante, P. et al., 2006).

The notion that separation from glycosaminoglycan containing domains or glycosaminoglycan removal is necessary to reveal the adhesive functions of perlecan domains is consistent with previous studies (Brown, J.C. et al., 1997; Hayashi, K. et al., 1992). These investigations also demonstrated that the presence or addition of heparan sulfate were dominant inhibitors of cell adhesion. Thus, heparanase action also should be considered as a modulator of cryptic adhesive functions of the perlecan core protein (Whitelock, J.M. et al., 1996). From a physiological standpoint, these events may occur during tissue remodeling or damage or morphogenesis during which such extracellular matrix modifying enzymes are produced. A consequence would be to alter the activity of perlecan at tissue boundaries, i.e., basal lamina, to release resident growth factors as well as convert perlecan and/or perlecan fragments from non-adhesive to adhesive factors to promote cell growth, differentiation and attachment. In this context, it is extremely interesting that cell adhesion to the active motif isolated from the

sequence of perlecan domain IV Ig module in this study was completely blocked by heparin, but not by chondroitin sulfate, which would be very rich in the territorial matrix of cartilage.

It has been suggested that $\beta 1$ integrins interact with perlecan or perlecan domain V (Brown, J.C. et al., 1997; Hayashi, K. et al., 1992). In endothelial cells, attachment to mouse perlecan can be completely blocked by a combination of $\beta 1$ and $\alpha \nu \beta 3$ integrin function blocking antibodies (Hayashi, K. et al., 1992). These observations are consistent with the occurrence of an RGD motif in the mouse protein; however, because the human protein lacks this motif, it is not clear if these same receptors are functional in perlecan-dependent adhesion events in humans. Our combined findings that adhesion was only partially affected by antibody blockade of $\beta 1$ integrins and was divalent cation independent suggests that non-integrin receptors mediate attachment to the novel perlecan domain IV peptide sequence. In this regard, the location of the peptide motif in an Ig loop suggests that interactions with Ig superfamily members may be involved (Walsh, F.S. and Doherty, P., 1997). Because Ig superfamily receptors also may influence integrin-dependent events (Zell, T. et al., 1999), these interactions may be quite complex. We considered that cell surface receptor binding to perlecan domain IV peptide was indirect and mediated by a second extracellular protein that bound both to the peptide as well as one or more cell surface receptors. The only protein known to interact with the region of perlecan containing the peptide IV motif is fibulin-2 (Hopf, M. et al., 2001). We attempted to determine if peptide IV interacted with fibulin-2, but were unable to demonstrate stable binding to the peptide in solid phase binding assays or inhibition of cell adhesion to peptide-4 by fibulin-2 antibodies (data not shown).

Regardless of the receptors involved, it is clear that adhesion to perlecan domain IV peptide triggers cytoskeletal reorganization (formation of filamentous actin) and FAK activation. Consequently, attachment to this motif results in signal transduction and by this criteria reflects a receptor-mediated event. Other approaches toward identifying functionally active motifs in proteins have relied on the generation of recombinant proteins containing deletions or point mutations in regions believed to be active, and indeed ongoing work in our laboratory will test the function of the Ig repeat region from domain IV. While this type of approach has been successful in many cases, it is nonetheless time-consuming, expensive and does not fully exploit the current availability of protein databases and structure/function predicting algorithms to identify biologically relevant peptide motifs. The present studies demonstrate the feasibility of utilizing a combined bioinformatics approaches with bioassays to rapidly screen for such motifs in large, complex proteins such as perlecan. These approaches are likely to be useful in identifying important peptide sequences in many other contexts. Even a 20% success rate using this approach will be able to identify large numbers of novel peptides for biochemical and tissue engineering studies.

Experimental Procedures

Cell Culture

C₃H10T1/2 murine fibroblasts and MG-63 human osteosarcoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM-F12). PC3 human prostate cancer cells were cultured in RPMI-1640 medium. HES, human uterine epithelial cells were grown in DMEM supplemented with 10% (w/v) charcoal-stripped fetal bovine serum FBS (Hyclone, Logan, UT) and sodium pyruvate solution. HS5 and HS27a human bone marrow stromal cells were cultured in DMEM with low glucose and no pyruvate. All media were supplemented with 10% (w/v) FBS and 100 units/mL penicillin and 100 μ g/mL streptomycin (PS) unless otherwise stated. Cells were maintained in T75 flasks (Corning Life Sciences, Lowell, MA) as described above until they reached 80% confluency. Afterwards, cells were harvested for cell adhesion assay. All cells were purchased from American Type Culture Collection (ATCC), and cell culture products were obtained from Invitrogen (Carlsbad, CA).

Bioinformatics analyses

The complete peptide sequences of human and murine perlecan were obtained from UniProtKB/Swiss-Prot entries P98160 (PGBM_HUMAN) and Q05793 (PGBM_MOUSE). Sequences were stored on the SDSC Biology Workbench (<http://workbench.sdsc.edu/>) and analyzed using various programs including GREASE (Kyte-Doolittle Hydropathy Profile). Five hydrophilic conserved peptide sequences were chosen for further analysis: EYRCDDRRPDCRDMSEDEL (Perlecan I; LDL-receptor class A 1 domain); GHCI PRDYLC DGQEDC (Perlecan II; LDL-receptor class A 2); CESCAPGYEGNPIQPGGK (Perlecan III; Laminin EGF-like 3); TWSKVGGHLRPGIVQSG (Perlecan IV; Ig-like C2-type 21 [human]; 14 [mouse]); LLFSGGKSGPVEDFVS (Perlecan V; Laminin G-like 2). All peptides were C-terminal of domain I which we have previously shown to be non-adhesive when decorated with glycosaminoglycan chains (French, M.M. et al., 2002). The first two peptides are from domain II, the third from domain III, the fourth from domain IV and the fifth from domain V. Domain structural designations are consistent with the Sanger database Pfam (www.sanger.ac.uk/Software/Pfam). Two dimensional analyses of peptide IV within the Ig-like repeat 14/21 followed the model of Hopf et al (Hopf, M. et al., 2001). Three dimensional models were predicted using 3D-PSSM (successor Phyre [<http://www.sbg.bio.ic.ac.uk/phyre/>]) and homology modeled to the immunoglobulin fold of PECAM/CD31 (Newton, J.P. et al., 1997).

Cyanuric Chloride Activation and Coupling

Covalink™ NH₂ plates (Nalge Nunc, Rochester, NY) were activated for five min at room temperature following the manufacturer's standard activation protocol. Following incubation in 100 µL of cyanuric chloride solution containing acetone, plates were washed three times with 300 µL of phosphate buffered saline (PBS, pH 7.0) to removal any residual solution. Plates then were dried at 50°C for 30 min and immediately sealed to store at room temperature until further use. Each peptide (10 µg) was added to the designated well and allowed to incubate overnight at room temperature. After incubation, the plates were washed with 300 µL of Covabuffer™, containing 2 M NaCl, 1% (w/v) MgSO₄, 0.05% (v/v) Tween 20 in PBS. The last wash was incubated for 5 min to ensure all residual unlinked peptide had been removed. Plates subsequently were blocked with 1% (w/v) heat denatured BSA for 1 hr at 37°C to block any residual sites for protein binding. Following blocking, plates were prepared for cell adhesion assay.

Peptide/BSA Conjugation

The protocol suggested by the manufacturer was followed for the Imject® Immunogen EDC Conjugation Kit (Pierce, Rockford, IL). EDC (10 mg) was added to a 700 µl solution containing 2 mg of peptide in conjugation buffer and 2 mg of BSA in water. The reaction mixture was incubated for 2 hr at room temperature in the dark. Following incubation, conjugated peptide was separated from unconjugated peptide using gel filtration with D-Salt™ Dextran Desalting Columns (Pierce). Columns were prepared according to manufacturer's protocol. In brief, columns were washed with 15–20 ml of purification buffer. Fractions were collected in purification buffer. Protein and peptide elution positions were monitored by A₂₈₀. Conjugated fractions eluting in the void column volume were pooled, syringe filtered for sterilization and stored at –20°C.

Mass Spectrometric Analysis

The conjugated peptide was analyzed using by SELDI-TOF mass spectroscopy using a Ciphergen® system (Fremont, CA), now part of BioRad (Hercules, CA). A normal phase NP20 hydrophilic protein chip was used in determining the conjugation efficiency. The NP20 chip was activated with 3 µL of water. After the water dried, 1 µL of the sample was added to each

spot on the chip, followed by another 1 μ L of sample to each spot. The air-dried sample was incubated on the chip for an additional 5–10 min. Each spot then was washed with 4 μ L of HPLC grade water and 50% (v/v) acetonitrile. After air-drying the chip, 1 μ L of saturated α -cyano-4-hydroxy cinnamic acid solution® was applied to each spot and allowed to dry for 1 min. The chip then was analyzed by SELDI-TOF.

Cell Adhesion Assay

Concentrated stocks of rat-tail type I collagen (Collaborative Research, Bedford MA) were diluted to 0.1 mg/ml in 0.02 N acetic acid and coated overnight on Maxisorp® 96 well plates at room temperature. Poly-L-lysine (PLL) (0.01 mg/ml), 3% (w/v) heat-denatured bovine serum albumin (BSA), perlecan domain IV peptide conjugated to BSA and scrambled perlecan domain IV peptide conjugated peptide to BSA (peptide BSA conjugates were added at 0.1 mg/ml) were diluted in phosphate buffered saline (PBS) and then used to coat Maxisorp® 96 well plates as described above. The following day, the plates were washed 3 times with PBS to remove any residual unadsorbed material and then blocked with 1% (w/v) heat-denatured BSA for 1 hr at 37°C. Following blocking, plates were washed once with PBS before use. For use in cell attachment assays, cells were detached with 0.5 mM EDTA and collected by centrifugation. Cells were resuspended in serum-free RPMI 1640 and plated at a concentration of 2.5×10^4 cells per 100 μ L of serum-free RPMI-1640. Cells then were allowed to attach to the wells for 1 hr at 37°C. Next, the wells were washed once with 1% (w/v) heat-denatured BSA to remove any unattached cells. Cell adhesion was quantified by measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to formazan according to the manufacturer's instructions (Promega, Madison, WI). To determine the influence of divalent cations, cell attachment assays were performed in the presence of solutions containing various combinations of free Ca^{2+} (2 mM), Mg^{2+} (2 mM), both ions (2 mM), no divalents or EDTA. For glycosaminoglycan inhibition studies, 3% (w/v) heat-denatured BSA and 0.1 mg/ml perlecan domain IV peptide-BSA conjugate were diluted in phosphate buffered saline (PBS) and used to coat Maxisorp® 96 well plates overnight as described above. The following day, the plates were washed 3 times with PBS to remove any residual unabsorbed material and then blocked with 1% (w/v) heat-denatured BSA for 1 hr at 37°C. Blocked plates were washed once with PBS. Heparin (porcine intestinal mucosa, sodium salt) (Sigma-Aldrich, St. Louis MO) or chondroitin sulfate (shark cartilage, sodium salt) (Sigma-Aldrich) at either 20 or 200 μ g/ml in a volume of 50 μ L of serum-free RPMI-1640 was added to wells coated with perlecan domain IV peptide and incubated for 15 min at 37°C. As a control, one set of wells containing peptide conjugate was not incubated with heparin nor were collagen (positive control) and BSA (negative control) wells. Immediately after incubation, cells (2.5×10^4) prepared as above in serum-free RPMI-1640 were added to all wells in a volume of 50 μ L. Cells were allowed to attach to the wells for 1 hr at 37°C, then the wells were washed once with 1% (w/v) heat-denatured BSA to remove any unattached cells. Cell adhesion was quantified by measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to formazan according to the manufacturer's instructions (Promega, Madison, WI).

Flourescence labeling

Eight well plates containing 10,000 cells/well were prepared as in the cell adhesion assay. The cells were fixed with 4% (w/v) paraformaldehyde for 10 min. Following fixation, excess aldehyde was quenched with 50 mM NH_4Cl for 15 min at room temperature. Fixed cells then were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min at room temperature. The plates then were blocked for 45 min with 3% (w/v) BSA. Alexa Flour® 488 phalloidin (Invitrogen) was added to the cells at a 1:100 dilution in PBS, and incubated for 40 min at 37°. After extensive washing in PBS, To-Pro®-3 iodide solution (Invitrogen) diluted 1:5000 in PBS was added and incubated for 10 min at room temperature. Cell layers were rinsed three times

with PBS and then visualized by confocal microscopy using a Zeiss LSM 510 VIS attached to an Axiovert 100M.

FAK activation

Cells were plated onto coated wells as described above for the cell adhesion assay then fixed in 4% (v/v) paraformaldehyde for 40 min. Cells were prepared for FAK staining as suggested by the manufacturer (Upstate Biotechnology, Lake Placid, NY now Millipore, Billerica, MA) except that PBS was used instead of TBS. Cells were permeabilized using 0.1% (v/v) Triton X-100 for 5 min at room temperature, then blocked with 1% (v/v) heat denatured BSA for 30 min. After wash, cells were incubated in 1:75 dilution of rabbit anti-phospho FAK (Y397) for 1 hr then washed twice in PBS prior to addition of secondary antibody (goat anti-rabbit IgG) for 30 min. Detection was with donkey anti-goat IgG cyanine conjugate tertiary antibody. Cells were examined using a fluorescence microscope equipped with a digital camera.

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Figure 1. Domain structure of murine perlecan showing location of perlecan domain IV peptide, TWSKVGGHLRPGIVQSG, in the fourteenth Ig-like repeat of domain IV
The diagram shows a schematic of perlecan indicating the five major domains (I–V) and the amino- (N) and C- (C) termini. Threadlike structures in domains I and V represent attached glycosaminoglycan chains. The location and sequence of the peptide used for most studies in this paper is indicated in the box. Similarities of various motifs in perlecan to other protein motifs are indicated in the inset. This figure was adapted from Olsen, 1999.

Figure 2. Hydrophilicity plot and secondary structure prediction of perlecan domain IV Ig module sequence

Hydrophilicity (A) was determined using the online program GREASE from the SDSC Workbench, the arrow indicates the hydrophilic region in which the chosen peptide is located. Secondary structure (B) was predicted based on the homology model of Hopf and colleagues as described in “Experimental Procedures”. The TWSKVGGHLRPGIVQSG sequence (white letters) is predicted to lie on the outside of the Ig module where it would be in contact with the aqueous solution and potentially interact with other proteins or surfaces.

Figure 3. 3-D structure prediction of perlecan domain IV Ig module (I-Set) with site of TWSKVGGHLRPGIVQSG peptide shown (arrow)

The tertiary structure of the Ig module containing the active peptide

TWSKVGGHLRPGIVQSG was predicted using 3D-PSSM (successor Phyre) giving a 100% estimated precision value for an Ig I-Set conformation. The arrow points to the position of the region containing the peptide projecting outward like a finger from the beta-sandwich structure.

Figure 4. C₃H10T1/2 fibroblast adhesion to perlecan domain specific peptides attached to Covalink™ plates

Peptides, conditions used for covalent attachment to Covalink™ plates and quantitation of cell adhesion assays are as described in “Experimental Procedures”. The peptide concentrations used during the initial peptide attachment step are indicated in the figure. In each sequence, the peptides designated I, II, II IV and V are arranged in order from left to right. Each bar represents the mean $\pm$ standard deviation of triplicate determinations from a representative experiment. Adhesion was highest to the perlecan domain IV peptide TWSKVGGHLRPGIVQSG.

Figure 5. Mass spectrometry of BSA conjugated perlecan domain IV peptide

Mass spectrometry was performed using a SELDI system as described in “Experimental Procedures”. The top panel shows the mass spectrum of the unconjugated domain IV peptide used in some of the current studies. The middle and bottom panels show the spectra of unconjugated BSA and BSA conjugated with perlecan domain IV peptide, respectively. Note that the mass spectrum of the BSA-peptide conjugate ranges from approximately 68,200, i.e., the mass expected after the addition of one 1780 peptide to the BSA conjugate (median mass 66,433), to approximately 92,000 with a median mass of 79,535. Thus, the median number of peptides conjugated to BSA in these preparations was 7 with a maximum of 14 peptides per BSA molecule. BSA conjugated peptides of these characteristics were used in most of the subsequent cell adhesion assays.

Figure 6. MG-63 osteoblastic cells adhere to perlecan domain IV peptide, but not scrambled domain IV peptide

Coating of surfaces with collagen, heat-denatured BSA and BSA-conjugated peptides as well as quantitation of cell adhesion were as described in “Experimental Procedures”. In the case of perlecan peptide IV (Pln IV) or scrambled perlecan peptide IV (Scr Pln IV), three different concentrations of BSA-conjugated peptide, i.e., 0.01 mg/ml, 0.1 mg/ml or 1 mg/ml, were used to coat surfaces before blocking with heat-denatured BSA. The PBS control represents binding to uncoated tissue culture plates. Type I collagen served as the positive binding control. The bars indicate the means $\pm$ S.D. of triplicate determinations in each case. *** $p < 0.001$ relative to BSA or scrambled peptide-BSA coated surfaces.

Figure 7. Cell type specificity of adhesion to perlecan domain IV peptide

Coating of surfaces with BSA-conjugated peptides as well as quantitation of cell adhesion were as described in “Experimental Procedures”. For each cell line, 2.5×10^4 cells were added per well to wells coated with either (0.1 mg/ml) perlecan domain IV peptide (Pln IV) or scrambled perlecan domain IV peptide (Scr Pln IV). The bars indicate the means $\pm$ S.D. of triplicate determinations in each case; when not seen they were so close as to be obscured. *** $p < 0.05$, ** $p < 0.01$; * $p < 0.001$ relative to adhesion observed using scrambled peptide for the respective cell line. HES = human endometrial line; MG63 = human osteoblastic line; PC3 = human prostate cancer line; HS5 and HS27a = human bone marrow stromal cell lines. HS5 and HS27a cells adhered well to tissue culture plastic or to type I collagen coated wells (data not shown).

Figure 8. MG63 osteoblastic cells spread and organize their cytoskeleton on perlecan domain IV peptide coated surfaces

Coating of cell surfaces with heat-denatured BSA (BSA), type I collagen, BSA-conjugated perlecan domain IV peptide (Pln IV) or BSA-conjugated scrambled domain IV peptide (Scr Pln IV) was as described in “Experimental Procedures”. The panels on the left (A–D) show the light microscopic images of cells bound to each indicated surface while the panels on the right (E–H) show the fluorescent images obtained of cells stained with the nuclear stain, TOPRO3 (blue) and FITC-phalloidin (green). Note that only cells plated on collagen type I or perlecan domain IV peptide cells displayed an organized actin cytoskeleton reflected by strong phalloidin staining at the cell periphery. Magnification is indicated in the top panel for each series.

Figure 9. FAK activation of C₃H10T1/2 cells occurs on perlecan domain IV peptide

Coating of surfaces with PLL, collagen, and BSA-conjugated perlecan domain IV peptide and assay of FAK activation at tyrosine 397 after one hr were performed as described in “Experimental Procedures”. As seen, FAK was activated in both collagen and perlecan domain IV peptide coated wells (bright white signal) compared to cells on PLL which showed lesser signal. The images from both BSA blocked and scrambled perlecan wells were blank. Note the degree of cell spreading on perlecan domain IV peptide which also was observed by phalloidin staining.

Figure 10. Divalent cation independence of MG-63 osteoblastic cell adhesion to perlecan domain IV peptide

Coating of surfaces with collagen, heat-denatured BSA and BSA-conjugated peptides as well as quantitation of cell adhesion were as described in "Experimental Procedures". The four bars on the left show the results obtained when the assays were performed for BSA, collagen type I, tissue culture plastic in PBS, or scrambled perlecan peptide IV (Scr). Binding to perlecan peptide IV under the standard adhesion assay conditions, i.e., in the presence of both Ca^{2+} and Mg^{2+} is shown in the middle. The four bars to the right indicate the results obtained when the assays were performed on perlecan IV-coated surfaces in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS or re-supplemented with 2 mM CaCl_2 (Ca^{2+}), 2 mM MgCl_2 (Mg^{2+}), 1 mM CaCl_2 plus 1 mM MgCl_2 ($\text{Ca}^{2+} + \text{Mg}^{2+}$) or no divalent cation supplement (2 mM EDTA only). The bars represent the means $\pm$ S.D. of triplicate determinations from a typical experiment.

Figure 11. Adhesion of C₃H10T1/2 cells to perlecan domain IV peptide is blocked by heparin, but not chondroitin sulfate

Coating of surfaces with collagen, heat-denatured BSA (BSA) and BSA-conjugated peptides (perlecan IV, Scr perlecan IV) as well as quantitation of cell adhesion in the presence of glycosaminoglycans were as described in “Experimental Procedures”. Cells adhered well to both collagen and perlecan domain IV peptide in the presence of chondroitin sulfate (20 and 200 μ g/ml) but adhesion was inhibited completely by heparin at both 20 and 200 μ g/ml. Bars represent means $\pm$ S.D. of quadruplicate determinations from a typical experiment. *** $p < 0.001$.