Alternative Splicing of the IIICS Domain in Fibronectin Governs the Role of the Heparin II Domain in Fibrillogenesis and Cell Spreading*

Received for publication, November 28, 2001, and in revised form, January 23, 2002
Published, JBC Papers in Press, February 6, 2002, DOI 10.1074/jbc.M111361200

Amy J. Santas‡§, Jennifer A. Peterson‡, Jennifer L. Halbleib‡, Sue E. Craig‡, Martin J. Humphries‡, and Donna M. Pesciotta Peters¶**

From the Departments of Pathology and Laboratory Medicine and Ophthalmology and Visual Sciences, University of Wisconsin, Madison, Wisconsin 53706 and the Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, M13 9PT Manchester, United Kingdom

The Heparin (Hep) II-binding domain of fibronectin regulates the formation of focal adhesions and actin stress fibers and hence plays an important role in cell spreading, migration, and fibronectin fibrillogenesis. Using human skin fibroblast cultures, we demonstrate that alternative splicing of the neighboring IIICS domain may regulate the activities of the Hep II domain in cell spreading and fibronectin fibrillogenesis. Recombinant Hep II domains, adjacent to either the IIICS domain or the H89 splice variant that contains the amino-terminal sequence of the IIICS domain, blocked fibronectin fibrillogenesis and required sulfated proteoglycans to mediate cell spreading. If the Hep II domain was adjacent to either the H0 or H95 splice variants, which both lack the amino terminus of the IIICS domain, fibrillogenesis was not inhibited and cell spreading was independent of a sulfated proteoglycan-mediated mechanism. The effect of the splice variants on the Hep II domain could be mimicked using a Hep II domain that contained only 6 amino acids from the III13 repeat or 10 amino acids from the IIICS domain suggesting that sequences proximal to the III14 repeat determined the role of the Hep II domain in these processes. We propose that alternative splicing of the IIICS domain modulates interactions between heparan sulfate proteoglycans and the Hep II domain and that this serves as a mechanism to control the biological activities of fibronectin.

As a major protein in the extracellular matrix, fibronectin provides positional cues to help direct a wide variety of biological processes including cell migration, adhesion, cell cycle progression, cell differentiation, and apoptosis (1, 2). These biological activities of fibronectin are mediated via interactions with various members of the integrin family and cell surface proteoglycans. It has been known for some time that these biological activities of fibronectin are contained within discrete structural domains and that neighboring domains within fibronectin can either suppress or enhance a particular activity. For example, the heparin (Hep)† II-binding domain of fibronectin can suppress the expression of metalloproteinases induced by the central cell-binding domain of fibronectin (3, 4), indicating that the various domains of fibronectin can act cooperatively to regulate the biological activity of fibronectin.

During transcription, four regions of fibronectin are alternatively spliced to generate up to 20 different isoforms of fibronectin (2, 5). Two of the alternatively spliced sites known as the extra type III repeats (EIIIA and EIIIB) flank the central cell-binding domain of fibronectin and alternative splicing results in either the exclusion, or inclusion of these domains. A third site of alternative splicing occurs within the IIICS domain (also known as the variable region). Alternative splicing of this domain results in either the inclusion, or exclusion, of this domain as well as the removal of only part of the amino and/or carboxyl termini of the domain (6). The fourth site of alternative splicing is located at the carboxyl termini of fibronectin and results in the removal of the III15 and I10 repeats (7).

Alternative splicing of fibronectin is tissue-specific. Fetal tissues and tumors express a higher percentage of fibronectins with the EIIIA and EIIIB repeats. Expression of the EIIIA and EIIIB isoforms is also increased during wound healing (8–11). Fibronectin isoforms that lack the III15 and I10 repeats are found exclusively in cartilage (7) and 50% of fibronectins in the plasma may lack the IIICS domain (12).

Alternative splicing can alter the activity of fibronectin by introducing a new activity that is contained within that spliced site. For example, alternative splicing of the IIICS domain will either add, or remove, integrin- and proteoglycan-binding sites thereby affecting cell adhesion or migration (13–16). In human periodontal ligament fibroblasts, inclusion of the entire IIICS domain modulates the activity of FAK and regulates apoptosis (17). Inclusion of the EIIIA repeat regulates the expression of metalloproteinases in joint connective tissues, mediates the induction of the myofibroblastic phenotype by transforming growth factor-β1, and activates lipocytes (18–20).

The IIICS domain and the EIIIA and EIIIB repeats also influence the biological activities of neighboring domains. Thus, the IIICS domain acts cooperatively with the Hep II domain of fibronectin to control the invasive phenotype of human oral squamous cell carcinoma (21). When the alternatively spliced EIIIA domain is included in the translated product, the

*This work was supported in part by National Institutes of Health Grant EY12515, the American Heart Association grant-in-aid, Wisconsin Affiliate, National Science Foundation Grant MCB9728382 (to D. M. F.), and the Wellcome Trust (to M. J. H.). 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 only to indicate this fact.

§Current address: Div. of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109.

**To whom correspondence should be addressed to: Dept. of Pathology and Laboratory Medicine, Rm 6590 MSC, 1300 University Ave., Madison, WI 53706. Tel.: 608-262-4626; Fax: 608-265-3301; E-mail: dmpeter2@facstaff.wisc.edu.

† The abbreviations used are: Hep II, heparin II; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; GST, glutathione S-transferase.

13650 This paper is available on line at http://www.jbc.org
cell adhesion function of the neighboring integrin-binding domain in the III\textsubscript{10} repeat is enhanced (22). In addition, the role of III\textsubscript{14} in cell cycle progression and mitogenic signal transduction is enhanced when the EIIIA repeat is included (23). Presumably insertion of the EIIIA domain enhances these functions because it introduces a global conformational change into fibronectin that further exposes the integrin-binding site in the III\textsubscript{14} repeat. A similar function may exist for the EIIIB repeat which when included within fragments from the central cell-binding domain enhances cell adhesion and spreading (24).

The Hep II-binding domain is sandwiched between the alternatively spliced EIIIA repeat and the IIICS domain and is therefore a likely candidate to be affected by alternative splicing. It plays an important role in regulating cell adhesion, migration, fibronectin fibrillogenesis, signal transduction events, and the organization of focal adhesions and the actin cytoskeleton (17, 21, 25–29). The Hep II domain contains binding sites for αβ1β3 integrins (30) and members of the syndecan family (28, 31). In this present study, we examined whether alternative splicing of the IIICS domain affects the biological activity of the Hep II domain. Using recombinant proteins that contain the Hep II domain and four splice variants of the IIICS domain (H0, H120, H89, or H95), we show that alternative splicing of the IIICS domain regulates the ability of soluble Hep II domains to block fibronectin fibrillogenesis and utilize sulfated proteoglycans to promote cell spreading. Alternative splicing of the IIICS domain did not compromise the ability of the Hep II domain to bind to either the amino terminus of fibronectin or the fibroblast cell surface. Alternative splicing, therefore, appears to be a mechanism to control the biological functions of the Hep II domain, possibly by modulating cell signaling pathways.

**MATERIALS AND METHODS**

**Cell Binding Assay**—Neonatal human skin fibroblasts used in the binding assays were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. All binding assays were done as previously described (29). Freshly trypsinized cells were resuspended in DMEM containing 2 mg/ml bovine serum albumin (BSA), 100 units/ml penicillin G, 5 μg/ml streptomycin sulfate, and 25 μg/ml cycloheximide and then incubated for 1 h at room temperature before being plated onto a 96-well plate at a final density of 3 × 10^4 cells/well. Prior to labeling with 125I-ligands, cells were allowed to adhere and spread for 3 h at 37 °C. Fibroblasts were then incubated with recombinant fibronectin or 70-kDa fragments for 1 h at 37 °C. All microtiter wells were precoated for 1 h with 40 μg/ml 70-kDa fibronectin fragment. The III\textsubscript{12} repeat of fibronectin for 2 h. Cells were allowed to attach for 1 h in DMEM with 10% fetal bovine serum, before the medium was replaced with serum-free medium (DMEM, 25 μg/ml HEPEs, pH 7.4, 2 mg/ml BSA, 25 μg/ml cycloheximide, 100 units/ml penicillin G, and 5 μg/ml streptomycin sulfate). After 3 h, this medium was replaced and cells were incubated overnight at 37 °C with serum-free medium containing 1 μg/ml (2 nm) or 3 μg/ml (6 nm) human plasma fibronectin with or without recombinant proteins. Cell layers were then washed two times with phosphate-buffered saline and fixed for 30 min at room temperature with 4% paraformaldehyde (pH 7.4) and 0.1 M phosphate-buffered saline to remove unattached cells. The attached cells were fixed with 4% paraformaldehyde and 0.1 M sodium phosphate buffer, pH 7.4, for 30 min and viewed under a phase microscope. Quantification of the degree of spreading was done by randomly counting the number of spread versus round cells. At least 100 cells/well of triplicate wells were counted.

**Solid Phase Binding Assays**—Microtiter wells precoated with 10 μg/ml 70-kDa fibronectin fragment were incubated with increasing concentrations of 125I-labeled recombinant proteins in DMEM containing 25 mM HEPES and 2 mg/ml BSA for 1 h at 37 °C as described previously (29). Wells were then washed three times, separated, and counted. The recombinant proteins used included the His-III\textsubscript{12} (2.8 × 10^5 cpm/pmol), III\textsubscript{13} (1.7 × 10^5 cpm/pmol), III\textsubscript{14} (1.3 × 10^5 cpm/pmol), His-III\textsubscript{14} (6.6 × 10^4 cpm/pmol), His-II\textsubscript{11032}–III\textsubscript{14} (3.5–8 × 10^4 cpm/pmol), III\textsubscript{12,14} (3.8 × 10^4 cpm/pmol), and doublet (9.8 × 10^3 cpm/pmol) containing IIICS domain (3.2 × 10^5 cpm/pmol) or IIICS domain (1.1 × 10^5 cpm/pmol).

**Competition Binding Assays**—were done as previously described (29). Briefly, microtiter wells precoated with 10 μg/ml 70-kDa fragment were incubated with 125I-labeled His-III\textsubscript{14} (1.6 × 10^5 cpm/pmol) in the presence or absence of unlabeled proteins for 1 h at 37 °C. Wells were then washed three times, separated, and counted. Nonspecific binding was measured in wells coated with 2 mg/ml BSA.

**Matrix Assembly Immunofluorescence Microscopy**—The ability of the recombinant fibronectin domains to inhibit fibronectin fibrillogenesis was assayed as previously described (29). Human fibroblasts were plated at confluence (5 × 10^4 cells/well) onto Teflon\textsuperscript{TM}-coated 12-well glass slides that were precoated with 20 μg/ml of the 160-kDa cell-binding fragment of fibronectin for 2 h. Cells were allowed to attach for 1 h in DMEM with 10% fetal bovine serum, before the medium was replaced with serum-free medium (DMEM, 25 μg/ml HEPEs, pH 7.4, 2 mg/ml BSA, 25 μg/ml cycloheximide, 100 units/ml penicillin G, and 5 μg/ml streptomycin sulfate). After 3 h, this medium was replaced and cells were incubated overnight at 37 °C with serum-free medium containing 1 μg/ml (2 nm) or 3 μg/ml (6 nm) human plasma fibronectin with or without recombinant proteins. Cell layers were then washed two times with phosphate-buffered saline and fixed for 30 min at room temperature with 4% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4. Polyclonal anti-fibronectin serum and donkey anti-rabbit IgG conjugated to rhodamine were used to label fibronectin fibrils as described (34). Antibodies were diluted 1:100 with 1% BSA, phosphate-buffered saline. Labeled fibronectin fibrils were analyzed by epifluorescence with a Nikon Optiphot microscope and digitized images were collected with a Photometrics Image Point CCD\textsuperscript{TM} camera and the Image Pro Plus\textsuperscript{TM} version 1.3 program.

**Fragments of Fibronectin**—Human plasma fibronectin, 70-kDa (I\textsubscript{19}–I\textsubscript{9}), III\textsubscript{12–14}, and III\textsubscript{13} fragments, were prepared from isolated human plasma fibronectin as previously described (35–37). Recombinant glutathione S-transferase (GST) fusion proteins of the Hep IIa (III\textsubscript{12–14}), III\textsubscript{12}, and III\textsubscript{14} repeats of fibronectin were prepared as described before using thrombin to cleave off the GST tag (29). The III\textsubscript{14} repeat includes three amino acids (QST) upstream of the III\textsubscript{12} repeat and spans amino acids Glu\textsubscript{1781} through Thr\textsubscript{1787}. Primers used to amplify the III\textsubscript{14} repeat were: sense primer, 5′-GAATTCGACTTCAGAGTGTTGACAC-3′; and antisense primer, 5′-CTCGAGCTTCAGAGTGTTGACACATGCG-3′. Primers used to amplify the III\textsubscript{13} repeat were: sense primer, 5′-GAATTCGACTTCAGAGTGTTGACACATGCG-3′; and antisense primer, 5′-CTCGAGCTTCAGAGTGTTGACACATGCG-3′. Primers used to amplify the III\textsubscript{12} repeat were: sense primer, 5′-GAATTCGACTTCAGAGTGTTGACACATGCG-3′; and antisense primer, 5′-CTCGAGCTTCAGAGTGTTGACACATGCG-3′. Primers used to amplify the III\textsubscript{12} repeat were: sense primer, 5′-GAATTCGACTTCAGAGTGTTGACACATGCG-3′; and antisense primer, 5′-CTCGAGCTTCAGAGTGTTGACACATGCG-3′.
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RESULTS

Alternative Splicing of the IIICS Domain Regulates the Ability of the III_{12-14} Repeats to Serve as Soluble Competitors of Fibronectin—Previous studies had shown that proteolytic fragments of fibronectin that contain the III_{12-14} repeats of the Hep II domain inhibit the incorporation of exogenously added plasma fibronectin into fibrils (29). To determine whether alternative splicing of the IIICS domain affects the biological activity of the Hep II domain, cultures were incubated with recombinant proteins containing the III_{12-14} repeats of the Hep II domain and different splice variants of the IIICS domain (Fig. 1). Fibril formation was then examined by immunofluorescence microscopy. As shown in Fig. 2A, cycloheximide-treated fibroblast cultures assemble exogenously added plasma fibronectin into fibrils (arrowheads). If the Hep IIa protein, which contains the III_{12-14} repeats and the first 10 amino acids of the IIICS domain, was added to the cultures, the assembly of exogenous plasma fibronectin into fibrils was inhibited (Fig. 2B). This biological activity of the Hep II domain in fibrillogenesis was regulated by the splice pattern of the IIICS domain. If cycloheximide-treated fibroblast cultures were incubated with H120 proteins that contain the Hep II domain and the entire 120 amino acids of the IIICS domain, fibronectin fibrillogenesis was blocked (compare Fig. 2, A and C). The only fibronectin observed in these cultures were aggregates of the plasma fibronectin (arrowheads). In contrast, if the H0 protein that lacks the IIICS domain was used, fibronectin fibril formation was not inhibited (Fig. 2D, arrowheads). The effect of the H120 protein on fibrillogenesis was not due to the biological activity of the IIICS domain, since the IIICS domain alone did not block fibrillogenesis (Fig. 2E). The effect of the IIICS domain on the activity of Hep II domain required that the IIICS domain be tandem with the Hep II domain. If the entire IIICS domain was added simultaneously with the H0 variant, fibronectin formation was still not inhibited (Fig. 2F). This suggests that the sequences adjacent to the III_{14} repeat influence the ability the Hep II domain to inhibit fibronectin fibril formation.

To demonstrate that the amino acid sequences immediately carboxyl-terminal to the III_{14} repeat can affect the biological activity of the Hep II domain during fibrillogenesis, the immunofluorescence microscopy assays were repeated using recombinant H95 and H99 proteins (Fig. 1). As shown in Fig. 2G, if the III_{14} repeat of the Hep II domain was adjacent to the H95 splice variant, which has the first 89 amino acids of the IIICS domain, the Hep II domain inhibited fibrillogenesis. However, if the H99 splice variant that lacked the first 25 amino acids of the IIICS domain was adjacent to the Hep II domain, the Hep II domain had no effect on fibrillogenesis (Fig. 2H). Therefore, the ability of the Hep II domain to block fibrillogenesis is not simply due to the lack or presence of the IIICS domain, but rather is a result of the sequences that are tandem to the III_{12-14} repeats.

The III_{15} repeat did not appear to have any affect on the activity of the IIICS domain in the H120 variant. Recombinant proteins containing the Hep II domain, the entire IIICS domain, but lacking the III_{15} repeat (Fig. 1), behaved like the

Fig. 1. Schematic diagram of the recombinant and proteolytic fibronectin domains. Monomer of plasma fibronectin consists of type I (rectangles), type II (ovals), and type III (numbered circles) structural repeats, and the alternatively spliced IIICS domain (CS). The repeats encoded in the recombinant proteins and the splice pattern of the IIICS domain are schematically shown. The wavy line after the Hep II protein indicates that it contains the first 10 amino acids from the amino terminus of the IIICS domain. The proteolytic 70-kDa fragment used in this study is underlined.

Fig. 2. Alternative splicing of the IIICS domain affects the activity of the Hep II domain. Cycloheximide-treated fibroblasts plated on the 160-kDa fragment of fibronectin were incubated overnight with 3 μg/ml plasma fibronectin alone (A) or in combination with molar equivalents (4.6 μM) of Hep IIa (B), H120 (C), H0 (D), IIICS (E), H0 and IIICS (F), H99 (G), H95 (H), and FN51 (I). All cultures were fixed and labeled with a rabbit anti-fibronectin serum followed by an anti-rabbit IgG conjugated to rhodamine as described under “Materials and Methods.” Fibronectin fibrils are identified with arrowheads. Arrows indicate aggregates of fibronectin. Bars represent 20 μm.
H120 splice variant and also blocked fibrillogenesis (Fig. 2F). This further indicates that it was the presence of the sequences adjacent to the III14 repeat that modulated the activity of the Hep II domain.

To further demonstrate that alternative splicing can affect the role of the Hep II domain in fibrillogenesis, a biochemical matrix assembly assay using 125I-labeled fibronectin was employed (43). In this assay, cycloheximide-treated fibroblasts were incubated with 125I-labeled fibronectin in the presence of unlabeled 10−6 M 70-kDa fragments, H0, or H120 splice variants. Data represent the average of three separate experiments. Error bars indicate the standard error of the mean.

Fig. 3. H120 but not H0 splice variants block binding of 125I-labeled fibronectin to matrix-deprived fibroblasts. Cycloheximide-treated fibroblasts (3 × 104 cells/well) were plated for 3 h at 37 °C on microtiter wells adsorbed with 4.9 μM of the 160-kDa fragment of fibronectin. Cultures were then incubated for 1 h with 14 fmol (2 × 105 cpm/well) of 125I-labeled fibronectin in the presence of unlabeled 10−6 M 70-kDa fragments, H0, or H120 splice variants. Data represent the average of three separate experiments. Error bars indicate the standard error of the mean.

Alternative Splicing Does Not Affect the 70-kDa Binding Activity of the Hep II Domain—It has been generally assumed that many of the fibronectin domains that inhibit fibrillogenesis do so because they block binding of the amino terminus of fibronectin to the cell surface (29, 45). Previous studies have indicated that the binding of the amino-terminal of fibronectin to the cell surface is the initial critical step in fibrillogenesis (44). To determine whether alternative splicing of the IIICS domain affected the role of the Hep II domain in fibrillogenesis by altering the amino-terminal binding activity of the III12–14 repeats, we first identified the amino-terminal-binding site in the III12–14 repeats.

As shown in Fig. 4, the III14 repeat of the Hep II domain appeared to contain the majority of activity responsible for inhibiting fibrillogenesis, since fibronectin fibrillogenesis was always reduced compared with control cultures when cycloheximide-treated fibroblast cultures were incubated with recombinant proteins containing the III14 repeat of fibronectin (III12–14, His-III13–14, and His-III14). In contrast, neither the III12 nor III13 repeats have a substantial effect on the formation of fibronectin fibrils (Fig. 4, C and D).

Solid phase binding assays show that the III14 repeat also contains the major amino-terminal-binding site in the Hep II domain, since recombinant proteins containing either the III14 repeat or the III13–14 repeats efficiently bound a 70-kDa amino-terminal fragment of fibronectin (Fig. 5A, ● and □). Binding of the III14 and III12–14 repeats to the 70-kDa fragments occurs in a dose-dependent fashion. Both proteins appear to bind equally well to the 70-kDa fragments, as no differences were detected between the ability of the His-III14 and His-III13–14 to bind to the 70-kDa fragments throughout the range tested. Most of this binding activity appeared to be due to interactions with the III14 repeat. This is illustrated by the fact that at the highest concentration tested (104 fmol), the recombinant III14 repeats bind about 54 times better than the III12 repeat (Fig. 5A, △) to adsorbed 70-kDa fragments. This suggests that while the III13 repeats may contain binding sites for the amino terminus of fibronectin, the primary binding site is located within the III14 repeat of the Hep II domain. At all concentrations tested, neither the His-III12 repeat (Fig. 5A, △) nor the III12 repeat (data not shown) demonstrated any detectable binding to the adsorbed 70-kDa fragment. Binding of the His-III14 repeat to 70-kDa fragments was not due to the histidine tag since the His-III12 repeat did not bind at any of the concentrations tested.

Competitive binding assays with 125I-labeled His-III14 confirmed the specific binding interaction between the His-III14 repeat and the 70-kDa fragment. As shown in Fig. 5B, recombinant proteins containing the III14 repeat were the best competitors for binding of 125I-His-III14 to adsorbed 70-kDa fragments (Fig. 5B). At a concentration of 0.27 μg/ml, the His-III14, His-III13–14, and III12–14 recombinant proteins each competed for 60–70% of the 125I-His-III14 binding to the 70-kDa fragment. The competition was dose dependent since 2.7 μg/ml of the recombinant proteins containing the III14 repeat competed for greater than 80% of the 125I-His-III14 binding to the 70-kDa fragment. This further indicates that the majority of the 70-kDa binding activity of the III12–14 repeats is mediated through the III14 repeat. In contrast, neither the His-III12 nor the III13 recombinant proteins had any effect on 125I-labeled His-III14 binding to adsorbed 70-kDa fragments.
FIG. 5. Recombinant proteins containing the III14 repeat bind to adsorbed 70-kDa fragments. A, wells coated with the 70-kDa fragment were incubated with increasing concentrations of 125I-labeled recombinant His-III14 (2.2 × 10⁴ cpm/pmol) ( ), His-III13 (1.0 × 10⁵ cpm/pmol) ( ), His-III11 (6.8 × 10⁴ cpm/pmol) ( ), or His-III12-14 (3.0 × 10⁵ cpm/pmol) ( ). Data represent triplicate measurements. B, microtiter wells which had adsorbed 70-kDa fragments were labeled with 32 nm 125I-labeled His-III14 (1.5 × 10⁵ cpm/well) in the absence or presence of unlabeled His-III13, His-III14, His-III13-14, or Hep IIa proteins. Recombinant proteins including the type III14 repeat were the most effective competitors for the amino-terminal-heparin II binding interaction. In all experiments, data represent the means of triplicate measurements. Non-specific binding to BSA-coated wells was subtracted from data in direct binding assays. Error bars indicate the standard error of the mean (S.E.).

Surprisingly, despite the fact that the III14 repeat contains the major amino-terminal binding activity and is adjacent to the IIICS in fibronectin, alternative splicing of the IIICS domain did not affect the 70-kDa binding activity of the Hep II domain. As shown in Fig. 6, there is no significant difference in the ability of the H120 and H0 proteins to bind adsorbed 70-kDa fragments. Both these recombinant proteins bind to adsorbed 70-kDa fragments as well as the Hep IIa protein (Fig. 6, ). The binding interaction with the 70-kDa fragments was specific for the III12-14 repeats, since the IIICS domain did not demonstrate binding to adsorbed 70-kDa fragments (Fig. 6, ). This suggests that alternative splicing of the IIICS domain has no effect on the binding interaction between the Hep II domain and the amino terminus of fibronectin.

Effect of Alternative Splicing of the IIICS Domain on the Cell Binding Activity of the Hep II Domain—Since the splice pattern of the IIICS domain had no affect on the ability of the Hep II domain to bind to the amino terminus of fibronectin, we investigated whether the splice pattern had any affect on the ability of the Hep II domain to interact with the cell surface. Previous studies have shown that the Hep II domain contains binding sites for members of the syndecan and integrin families (28, 30, 31). In addition, interactions with members of these families (in particular syndecan-2 and α6β1 integrins) have the capacity to modulate fibronectin fibrillogenesis (46, 47).

Cell surface binding interactions with the Hep II domain were quantified using 125I-labeled proteins containing the Hep II domain with and without an adjacent IIICS domain. These binding assays indicated that alternative splicing of the IIICS domain did not affect binding of the Hep II domain to cells pre-spread on the 160-kDa fragment of fibronectin. As shown in Fig. 7A, at 10⁻⁸ M the H0 proteins which contain the Hep II domain but lack a neighboring IIICS domain bind with similar apparent affinities to fibroblasts cultures as recombinant proteins which contain both the Hep II domain and the amino-terminal sequences of the IIICS domain (H120 and Hep IIa). Binding of the H120 splice variants to the cell surface was not mediated by the IIICS domain, since the IIICS domain (Fig. 7A) did not demonstrate significant binding to the fibroblast cell surface. Furthermore, both the recombinant III14 and III13-14 repeats bind the cell surface suggesting that the binding was mediated by at least the III14 repeat. Whether the III13 repeat plays a role was not determined. The III12 repeat was not involved since it failed to show any significant binding to the cell surface (Fig. 7B).

The presence of the IIICS, however, does modulate interactions between cell surface-sulfated proteoglycans and the Hep II domain during cell spreading. To demonstrate this, fibroblasts were treated with chlorate to prevent sulfation of cell surface proteoglycans. Chlorate-treated cells were then plated on dishes pre-coated with either H0 or the Hep IIa proteins to determine the affect of alternative splicing on binding interactions between sulfated proteoglycans and the Hep II domain during cell spreading. As shown in Fig. 8A, fibroblasts efficiently spread on the Hep IIa protein. This cell spreading is mediated by interactions with sulfated proteoglycans, since chlorate-treated cells were able to attach but not spread on the Hep IIa protein. Approximately 80% of the cells on the Hep IIa protein remained round (Fig. 8B). This effect on cell spreading was reversible and cultures incubated with sulfate recovered their ability to spread on the Hep IIa protein.

As in fibrillogenesis, the sequences proximal to the III14 repeat affects the role of the Hep II domain during cell spreading. Whereas cell spreading on the Hep IIa protein is mediated...
by interactions with sulfated proteoglycans, cell spreading on the H0 variant that contained the III12–15 repeats was independent of sulfated proteoglycans. Approximately 80% of the chlorate-treated cells were able to spread on H0. In contrast, only 20% of the chlorate-treated cells could spread on the Hep IIa protein (Fig. 8B). This suggests that alternative splicing of the IIICS domain may affect interactions between sulfated proteoglycans, especially heparan sulfate proteoglycans, and the Hep II domain.

To test this hypothesis, chlorate-treated cells were also plated on the H120 splice variant that contained the entire IIICS domain. As shown in Fig. 8A, chlorate-treated cells spread poorly on the H120 splice variant compared with cells plated on the H0 splice variant and almost 80% of the cells were round. In contrast, cells were well spread on the H0 splice variant. Cell spreading on the H120 splice variant was therefore similar to cell spreading on the Hep IIa protein and is dependent on interactions with sulfated proteoglycans. A similar result was obtained when chlorate-treated fibroblasts were plated on the H89 splice variants (data not shown). In contrast, when chlorate-treated fibroblasts were plated on the H89 splice variant, the fibroblasts spread well indicating that this variant behaved like the H0 splice variant and cell spreading was now independent of interactions with sulfated proteoglycans (data not shown). Curiously, even though similar numbers of cells treated with chlorate and sulfate could spread on the H0, H120, and Hep IIa splice variants, cells treated with chlorate and sulfate did not appear to spread as well on the H120 splice variant as they did on H0 or Hep IIa splice variants (Fig. 8, A and B). The reason for this is unclear.

**Sequences Proximal to the Carboxyl Terminus of the III14 Repeat Regulate Its Activity in Fibrillogenesis and Cell Spreading**—To demonstrate further that the sequences adjacent to the carboxyl terminus of the III14 repeat in the Hep II domain regulates the activity of the Hep II domain in fibrillogenesis and cell spreading, a second recombinant III12–15 module (Hep IIb) was constructed. The Hep IIb protein differed from the Hep IIa protein in that it contained the first 6 amino acids of the III15 repeat instead of the first 10 amino acids of the IIICS domain (Fig. 9). As shown in Fig. 10, the Hep IIb protein in that it contained the first 6 amino acids of the III14 repeat instead of the first 10 amino acids of the IIICS domain (Fig. 9). As shown in Fig. 10, the Hep IIb protein had the opposite activity of the Hep IIa protein. Thus, in cycloheximide-treated fibroblast cultures the Hep IIa protein blocked fibronectin fibrillogenesis, whereas the Hep IIb protein did not (compare panels in Fig. 10, B and C). Cell spreading on the two Hep II proteins was also different. In chlorate-treated cultures, only 20% of chlorate-treated fibroblasts could spread on the Hep IIa protein (Fig. 8B). In contrast, over half (59%) of the fibroblasts could spread on the Hep IIb protein. Since this was the same percentage of fibroblasts as untreated fibroblasts that could spread on the Hep IIb protein, this indicates that cell
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spreading also differed between the Hep IIa and Hep IIb proteins. In bated with 4.6 μM sulfate (49). The Hep IIa protein. Cycloheximide-treated fibroblast cultures incu-

FIG. 9. Schematic diagram of the Hep IIa and Hep IIb proteins. The III12–14 repeats are represented by the numbered ovals. The IIICS domain and III15 repeat are indicated by the double arrow lines. The beginning amino acid sequences of the IIICS domain and the III15 repeat contained within the Hep IIa and Hep IIb proteins are indicated. The dashed line represents the sequences in the IIICS domain and III15 repeat not contained within the Hep II proteins. The Hep IIa protein consists of the III12–14 repeats and the first 10 amino acids of the IIICS domain. The Hep IIb protein consists of the III12–14 repeats and the first 6 amino acids of the III15 repeat.

spreading on Hep IIb protein was not affected by the chlorate treatment (compare Fig. 10, D and E). Thus, cell spreading on Hep IIb protein was similar to that on the H0 and H95 splice variants and was independent of interactions with sulfated proteoglycans. Therefore, the amino acids carboxyl-terminal to the III14 repeat determine the role of Hep II domain in fibrillogenesis and cell spreading.

FIG. 10. Sequences proximal to the carboxyl terminus of the III14 repeat determine the role of the Hep II domain in fibrillo-
genesis and cell spreading. The Hep IIb protein has the opposite effect on fibronectin fibrillogenesis (A–C) and cell spreading (D–F) than the Hep IIa protein. Cycloheximide-treated fibroblast cultures incubated with 4.6 μM of the Hep IIb protein (C) formed a fibronectin matrix that was indistinguishable from the matrix formed in the absence of any Hep II proteins (A). In contrast, fibronectin fibrillogenesis was inhibited in cultures incubated with 4.6 μM Hep IIa protein (B). Cell spreading also differed between the Hep IIa and Hep IIb proteins. In wells coated with 0.12 μM Hep IIb protein (D–F), chlorate-treated cells (E) spread to the same degree as untreated cells (D) or cells treated with sulfate (F). Bar represents 50 μM.

spreading on Hep IIb protein was not affected by the chlorate treatment (compare Fig. 10, D and E). Thus, cell spreading on Hep IIb protein was similar to that on the H0 and H95 splice variants and was independent of interactions with sulfated proteoglycans. Therefore, the amino acids carboxyl-terminal to the III14 repeat determine the role of Hep II domain in fibrillogenesis and cell spreading.

DISCUSSION

In this paper we show that alternative splicing of the IIICS domain can affect the biological activity of Hep II domain in fibronectin fibrillogenesis and cell spreading. When splice vari-

In this paper we show that alternative splicing of the IIICS domain can affect the biological activity of Hep II domain in fibronectin fibrillogenesis and cell spreading. When splice vari-

FIG. 10. Sequences proximal to the carboxyl terminus of the III14 repeat determine the role of the Hep II domain in fibrillo-
genesis and cell spreading. The Hep IIb protein has the opposite effect on fibronectin fibrillogenesis (A–C) and cell spreading (D–F) than the Hep IIa protein. Cycloheximide-treated fibroblast cultures incubated with 4.6 μM of the Hep IIb protein (C) formed a fibronectin matrix that was indistinguishable from the matrix formed in the absence of any Hep II proteins (A). In contrast, fibronectin fibrillogenesis was inhibited in cultures incubated with 4.6 μM Hep IIa protein (B). Cell spreading also differed between the Hep IIa and Hep IIb proteins. In wells coated with 0.12 μM Hep IIb protein (D–F), chlorate-treated cells (E) spread to the same degree as untreated cells (D) or cells treated with sulfate (F). Bar represents 50 μM.

Alternative splicing of the IIICS domain may be a mechanism to influence signaling events between cell surface heparan surface proteoglycans and the Hep II domain of fibronectin. The Hep II domain contains at least two sites where interactions with heparan sulfate proteoglycans can occur; one within the III13 repeat and another within the III14 repeat (45–53). Both of these heparin-binding sites modulate the formation of focal adhesions and stress fibers (25, 28, 54) and therefore play an important signaling role in regulating cell spreading. Alternative splicing of the IIICS domain may affect this role, since the H0 and H98 variants as well as the Hep IIb protein could promote cell spreading in the absence of cell surface heparan

amino acids of the IIICS domain supported identical biological activities as did the H120 and H89 variants. Not all biological activities of the Hep II domain were affected by alternative splicing of the IIICS domain. Neither the ability of the III12–14 repeats to bind to the fibroblast cell surface (28, 30) nor the ability to bind the amino terminus of fibronectin was affected (29). This suggests that alternative splicing of the IIICS do-

It is doubtful that alternative splicing of the IIICS domain or the 10 amino acids from the IIICS domain in the truncated Hep IIa domain altered the biological activities of the Hep II domain because it introduced different global conformational changes in the III12–14 repeats. CD spectra of the Hep IIa domain and the H0 variants were similar, indicating that the absence of the IIICS domain or the presence of just 10 amino acids from the IIICS domain did not significantly affect the folding of the III12–14 repeats (data not shown). Furthermore, not all the biological activities of the Hep II domain were affected by alternative splicing of the IIICS domain. All splice variants tested bound equally well to the amino terminus of fibronectin. This is unlikely to occur if major conformational changes had been introduced into the Hep II domain.

The IIICS splice variants and the amino acids at the carboxyl termini of the Hep IIa and IIb proteins could conceivably affect the biological activity of the Hep II domain by influencing interactions between the individual repeats of the Hep II domain. Thermal denaturation studies have indicated that there are strong cooperative interactions between the III13–14 repeats and that the III12–14 repeats act as a structural unit, even in the presence of 6 M urea (48). These interactions apparently influence the biological activity of the Hep II domain. Individual III13 and III14 modules bind heparin much less strongly than the parent fragments containing them (49) and as shown in this study the amino-terminal binding activity of the intact III12–14 repeats and their effectiveness as an inhibitor of fibrillo-
genesis differed from that of the individual III12, III13, and III14 repeats. Interestingly, these interactions had the same affect on the heparin binding activity of the III14 repeat and its ability to inhibit fibrillogenesis. That is, these activities were always stronger when the repeats were associated within the intact Hep II domain. In the case of the heparin binding activities of the III13 and III14 repeats this may be due to the precise juxtapositioning of the III13 and III14 repeats this may be due to the precise juxtapositioning of the III13 and III14 repeats (39). A similar situation may also exist for the role of the III14 repeat in fibrillogenesis, since fibrillog-
genesis has been proposed to involve the heparin binding activity of the Hep II domain (46, 50). Curiously, these interactions had the opposite affect on the amino-terminal binding activity of the Hep II domain. Why these interactions would have the opposite affect on the amino-terminal binding activity of the Hep II domain is difficult to reconcile without more information on the structural requirements needed for the binding of the amino terminus to the III12–14 repeats.

Alternative splicing of the IIICS domain may be a mecha-

nism to influence signaling events between cell surface heparan surface proteoglycans and the Hep II domain of fibronectin. The Hep II domain contains at least two sites where interactions with heparan sulfate proteoglycans can occur; one within the III13 repeat and another within the III14 repeat (51–53). Both of these heparin-binding sites modulate the formation of focal adhesions and stress fibers (25, 28, 54) and therefore play an important signaling role in regulating cell spreading. Alternative splicing of the IIICS domain affected this role, since the H0 and H98 variants as well as the Hep IIb protein could promote cell spreading in the absence of cell surface heparan
sulfate proteoglycans, whereas the Hep IIa protein and the variants H95 and H120 splice variants could not. This effect was not due to differences in the ability of H0 splice variant and the Hep IIa proteins to bind to the cell surface. In direct binding assays with radiolabeled ligands, H0, H120, and Hep IIa proteins bound equally well to the cell surface. This suggests that alternative splicing of the IICS domain modulates the ability of the Hep II domain to interact with heparan sulfate proteoglycans and that alternative splicing of the IICS domain may be a mechanism to restrict, or limit, the receptor-mediated signaling pathways used by the Hep II domain to control cell spreading so as to avoid unwarranted interactions.

The cell-surface-binding site used by the H0 variant to promote cell spreading in this study is not known. Several reports have now indicated that adhesion and spreading may involve cooperative interactions between cell surface proteoglycans and integrins, especially αβ1 integrins (41, 55, 56). Alternative splicing appears to affect cooperative interactions between the αβ1 integrin and heparan sulfate proteoglycan-binding sites in the IICS domain. In studies with A375-SM melanoma cells, both cell attachment and cell spreading on the H120 and H89 splice variants was almost exclusively dependent on αβ1 integrins, whereas cell attachment and spreading on the H0 and H95 splice variants involved cooperative interactions between αβ1, αβ1, and cell-surface proteoglycans (41). The Hep II domain is similar to the IICS domain in that it also has an αβ1, integrin binding site (15, 30, 39, 57) and heparin-binding sites (51–53, 56). Thus, it is conceivable that alternative splicing is regulating the ability of the Hep II domain to promote cooperative interactions between heparan sulfate proteoglycans and αβ1 integrins. In the case of the Hep IIa protein and the H120 and H89 splice variants, cell spreading may utilize cooperative interactions between the cell surface proteoglycan and αβ1 integrin-binding domains. Whereas, cell spreading on the Hep IIb protein, and the H0 and H95 splice variants may only require interactions with integrin signaling pathways.

It is clear from these studies that the amino-terminal binding activity of the Hep II domain may not be the major role of these repeats in fibrollogenesis as previously suggested (29). Alternative splicing did not affect the ability of the Hep II domain to bind to the amino terminus of fibronectin, but it did affect the ability of the Hep II domain to inhibit fibrollogenesis. Thus, there was not a direct correlation between the amino-terminal binding activity of the Hep II domain and the ability to block fibrollogenesis. The I1,14 and I13,14 repeats, which had the highest affinity for the amino terminus of fibronectin, were less effective at inhibiting fibril formation than the Hep IIa protein which contained the I12,14 repeats.

The splice variants that enable the Hep II domain to block fibrollogenesis also require interactions with sulfated proteoglycans to promote cell spreading. Therefore, the Hep II domain may be inhibiting fibrollogenesis not by binding to fibronectin directly but through signaling pathways initiated via interactions with cell surface proteoglycans. Indeed syndecan-2, a heparan sulfate proteoglycan found in fibroblasts that binds to the Hep II domain has been shown to have a role in fibrollogenesis (46, 50). When Chinese hamster ovary cells were transfected with a full-length syndecan-2, fibronectin fibrollogenesis was enhanced, but when the cells were transfected with syndecan-2 containing a truncated cytoplasmic domain, matrix assembly was blocked. Syndecan-2 appears to have a regulatory role in fibrollogenesis, since the transfection of the truncated syndecan-2 can override the induction of fibril formation via activated integrins (46). Thus, the Hep II domain may be mediating fibrollogenesis via a syndecan-mediated signaling pathway and not via a direct fibronectin-fibronectin binding interaction as previously suggested (29).

In conclusion, alternative splicing of the IICS domain not only affects cell-mediated adhesion events via the IICS domain but it also affects how isofoms of fibronectin utilize the Hep II domain in fibrollogenesis and cell spreading. Fibronec-"
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