

Cooperative Role for Activated $\alpha 4\beta 1$ Integrin and Chondroitin Sulfate Proteoglycans in Cell Adhesion to the Heparin III Domain of Fibronectin

IDENTIFICATION OF A NOVEL HEPARIN AND CELL BINDING SEQUENCE IN REPEAT III5*

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We recently reported that the heparin (Hep) III domain of fibronectin contains the H2 cell adhesion site in repeat III5 which binds activated $\alpha 4$ integrins. We have now further characterized the heparin and cell binding activities of this domain. A recombinant fragment containing repeats III4–III5 (FN-III4–5) induced Jurkat cell adhesion upon integrin activation with Mn^{2+} or TS2/16 monoclonal antibody (anti- $\beta 1$). Adhesion of Mn^{2+} -treated cells to FN-III4–5 or FN-III5 fragments was inhibited by chondroitinase ABC and ACII but not by the anti- $\alpha 4$ monoclonal antibody HP2/1. In contrast, HP2/1 completely blocked adhesion of TS2/16-treated cells while chondroitinase had a partial (FN-III4–5) or minor (FN-III5) effect. Thus, the role of each receptor depended on the stimulus used to activate $\alpha 4\beta 1$. The combination of HP2/1 and chondroitinase at dilutions which did not inhibit when used individually abolished adhesion of Mn^{2+} or TS2/16-treated cells to both fragments, indicating a cooperative effect between $\alpha 4\beta 1$ and chondroitin sulfate proteoglycans (CSPG). Furthermore, we have identified a 20-amino acid sequence in III5 (HBP/III5) which binds heparin and induces cell adhesion via CSPG exclusively. Although soluble HBP/III5 was a poor inhibitor, when combined with H2, it abolished adhesion to FN-III4–5 and FN-III5 fragments. These results establish that adhesion to the Hep III domain involves the cooperation of activated $\alpha 4\beta 1$ and CSPG and show that HBP/III5 is a novel heparin and CSPG-binding site contributing to cell adhesion to this domain.

Fibronectin (Fn)¹ is a plasma and extracellular matrix protein which interacts with other macromolecules and with cells

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¹ The abbreviations used are: Fn, fibronectin; CS, chondroitin-sulfate; HS, heparan sulfate; PG, proteoglycan; GAG, glycosaminoglycan; HBP/III5, heparin-binding peptide in repeat III5; CSPG, chondroitin sulfate proteoglycan; mAb, monoclonal antibody; KLH, keyhole limpet hemocyanin; Hep, heparin.

via specific binding sites present in well defined structural and functional domains (reviewed in Ref. 1). Fn contains two main cell adhesion domains located in the central and COOH-terminal regions, respectively. The active sites in the central domain are RGD in repeat III10 and its synergistic sequence PHSRN in repeat III9 (Refs. 1 and 2, see Fig. 1). These sites bind mainly $\alpha 5\beta 1$ integrin although RGD is also a ligand for activated $\alpha 4\beta 1$ (3). The COOH-terminal cell-binding region comprises the active sites CS-1 and CS-5 within the alternatively spliced segment IIICS, as well as H1 (IDAPS) in the high affinity heparin-binding domain or Hep II (Fig. 1). CS-1, CS-5, and H1 are ligands for $\alpha 4\beta 1$ integrin (4–9).

Besides the H1 $\alpha 4\beta 1$ -binding site, the Hep II domain contains several well characterized sequences which bind heparin and cell surface proteoglycans (PG) (10–12). One of these sequences is WQPPRARITGY (peptide FN-C/H V) (12) which mediates cell adhesion via PG and promotes focal adhesion formation (13, 14). There is now extensive evidence showing that PG may modulate the function of $\alpha 4\beta 1$ and that cell adhesion to the COOH-terminal region of Fn involves the cooperation between both types of receptors (11, 15–18). It is also well established that integrin function can be up-regulated by external factors including the divalent cation Mn^{2+} and certain anti- $\beta 1$ mAbs such as TS2/16 (19). Whether these reagents are mimicking the effects of physiologic regulators such as PG remains to be determined.

These previous studies have clearly established an important role for the Hep II domain of Fn in the adhesion of many cell systems including melanoma (11), lymphoid (5, 20), hematopoietic precursors (15, 18), and neural crest cells (21). However, it is now becoming evident that other heparin-binding regions also contribute to cell adhesion. Fn contains 2–3 additional heparin-binding domains located at the NH₂-terminal (Hep I) and central (Hep III) part of the molecule (see Fig. 1). These domains differ in their binding affinity and sensitivity to divalent cation regulation (22–24). The Hep I domain was recently shown to induce cell adhesion via interaction with the $\alpha 5\beta 1$ integrin (25). It is not known if this region also interacts with cell surface PG, although it binds several uncharacterized molecules at the cell surface (26).

We have also recently reported that a recombinant fragment containing the Fn III5 repeat, which is part of the Hep III domain, mediates lymphoid cell adhesion due to the interaction of the KLDAPT sequence with $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins previously activated with TS2/16 mAb or Mn^{2+} , respectively (27). Our previous observations therefore reveal a novel function for this domain and highlight the importance of heparin-binding

regions for the overall cell binding activity of Fn.

In the present study we have further characterized the heparin and cell binding properties of the Hep III domain of Fn. By preparing recombinant fragments containing type III repeats from this region, we show that adhesion of Jurkat T cells to a fragment containing Fn III4-III5 repeats involves the cooperation of activated $\alpha\beta 1$ integrin and chondroitin sulfate (CS) but not heparan sulfate (HS) PG. We also show that the contribution of each receptor depends on whether Mn^{2+} or TS2/16 are used to activate $\alpha\beta 1$ and that CSPG is crucial when Mn^{2+} is the reagent of choice. Furthermore, we have identified a novel 20-amino acid sequence in repeat III5, structurally similar to FN C/H V, which binds heparin and induces cell adhesion via CSPG exclusively. These results therefore establish novel interactions that regulate cell adhesion to the Hep III Fn domain.

EXPERIMENTAL PROCEDURES

Fibronectin Fragments and Synthetic Peptides—Recombinant fragments containing type III homology repeats 4–5–6 (FN-III4–5–6), 4–5 (FN-III4–5), 4 (FN-III4), and 5 (FN-III5) were produced by polymerase chain reaction amplification using UITma DNA-polymerase (Perkin-Elmer), cDNA from FN-III_{1/2}–11 clones and appropriate primers as described previously (27). Polymerase chain reaction products were cloned into pQE-12 or pQE-3/5 vector using the QIAexpress kit and expressed in *Escherichia coli*. All cloned cDNAs were sequenced using a Sequenase 2.0 DNA sequencing kit (U. S. Biochemicals Corp., Cleveland, OH). Fragments were purified by immunoaffinity chromatography using the specific mAbs (see below) IST-3 (FN-III4, FN-III4–5, and FN-III4–6) or IST-4 (FN-III5) conjugated to Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden). None of the fragments contained a 6xHis tag. Purity of recombinant fragments was confirmed by SDS-polyacrylamide electrophoresis gels (15% acrylamide; see Fig. 1). The recombinant fragment H0, comprising the Hep II domain (repeats III12–14) and repeat III15 was prepared exactly as described (28).

The synthetic peptides KLDAPT (H2), WTPPRAQITGYRLTVGLTRR (HBP/III5), WTPQARPITGYRLTVGLTRR (scrambled HBP/III5 or HP/III5.sc), WTPPRAQITGY (HBP/III5-N11), WTPPRAQITGYRLT (HBP/III5-N14), YRLTVGLTRR (HBP/III5-C10), and YQLTVGLTRR (HBP/III5-C10.sc) were synthesized on an automated multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany) using standard solid phase procedures and purified by reverse phase high performance liquid chromatography. Peptides were covalently conjugated to keyhole limpet hemocyanin (KLH, Calbiochem-Novabiochem Int., La Jolla, CA) with glutaraldehyde (29) by mixing KLH and peptide at a molar ratio of 1:3000 in phosphate-buffered saline. Glutaraldehyde was added dropwise to the peptide-KLH solution and the mixture incubated at room temperature for 2 h with gentle stirring; after extensive dialysis against phosphate-buffered saline, peptide coupling efficiency was determined by amino acid analysis after hydrolysis using a Biochrom 20 analyzer (Pharmacia).

Antibodies and Enzymes—IST-3 and IST-4 mAbs reactive with FN-III4 and FN-III5 repeats, respectively, were produced as reported (30). Activating anti- $\beta 1$ mAb T2/16 (purified Ab) and function blocking anti- $\alpha 4$ HP2/1 (culture supernatant) were generously donated by Dr. Francisco Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Chondroitinase ABC (EC 4.2.2.4) and heparinase III (heparitinase I, EC 4.2.2.8) were purchased from Sigma. Chondroitinase AC II (EC 4.2.2.5) and heparitinase (a mixture of 95% heparitinase I and 5% heparitinase II, EC 4.2.2.8) were purchased from Seikagaku America Inc. through ams Biotechnology (Oxon, United Kingdom).

Cells and Cell Cultures—The human T cell line Jurkat was obtained from Dr. Margarita López-Trascasa (Hospital La Paz, Madrid). Cells were maintained in RPMI 1640, 10% fetal bovine serum (ICN Pharmaceuticals, Costa Mesa, CA), and 24 μ g/ml gentamycin (Life Technologies, Inc., Middlesex, UK).

Cell Adhesion Assays—Flat bottom 8-well strips with N-oxy succinimide amine binding surface (Costar Co., Cambridge, MA) were coated overnight with recombinant fragments or peptides diluted in 0.1 M sodium borate, pH 8.5. Adhesion assays were carried out as described (3, 5, 20) for 3 h at 37 °C; attached cells were stained with toluidine blue and the absorbance at 620 nm was determined on a Multiskan Bichromatic plate reader (Labsystems, Helsinki, Finland). Quantitation of cell attachment was done using calibration curves as described (31) and optical density at 620 nm was found to be practically a linear function of the number of cells attached. Total cellular input was calculated in

these assays by spinning wells with the original number of cell aliquots, then fixing, staining, and measuring optical density. Integrin activation was performed by incubating the cells (total volume 250 μ l) with 5 μ l of TS2/16 mAb (0.28 mg/ml) in RPMI 1640, 10 mM Hepes, 1% bovine serum albumin, or 10 mM Tris, 150 mM NaCl, 1% bovine serum albumin, 2 mM Mn^{2+} , pH 7.2, for 15 min at 37 °C prior to the attachment assay. For inhibition experiments, cells were incubated in a total volume of 300 μ l, with appropriate dilutions of HP2/1 supernatant (1:5 or 1:50), chondroitinase ABC (0.3 or 1.0 Sigma units/ml), chondroitinase AC II (1 Seikagaku units/ml), heparinase III (0.3 or 1 Sigma units/ml), heparitinase (2 Seikagaku milliunits/ml), or synthetic peptides (0.5 mg/ml) for 30 min at 37 °C on a rotary shaker; the cell suspension was then diluted to 7×10^5 cells/ml and 100 μ l were added to each substrate-coated well. The assay was then continued as explained above. To rule out the possibility of protein degradation by protease contaminants present in the enzyme preparations, digestions were performed in the presence of 2 mM phenylmethylsulfonyl fluoride, 0.36 mM pepstatin A, 2.5 μ g/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor, and 10 μ g/ml aprotinin.

Heparin-Sepharose Affinity Chromatography—Recombinant Fn fragments were applied to columns containing heparin-Sepharose (Pharmacia) in 25 mM Tris, 30 mM NaCl, 0.5 mM Na₂EDTA, pH 7.4, buffer. After washing the columns with this buffer, bound fragments were eluted with a linear gradient ranging from 30 to 450 mM NaCl. Synthetic peptides (1 mg each) were dissolved in 0.4 ml of 25 mM Tris, 50 mM NaCl, 0.5 mM Na₂EDTA, pH 7.6, buffer and incubated with 0.3 ml of heparin-Sepharose matrix for 1 h at room temperature under mild shaking. Unbound material was determined by reading the absorbance of the solution at 280 nm after centrifugation. The beads were washed with the same buffer until the absorbance was zero and bound peptides were eluted using 25 mM Tris, 500 mM NaCl, 0.5 mM EDTA, pH 7.6, buffer and monitored by optical density at 280 nm. Further identification of the bound material was achieved by amino acid analysis and mass spectrometry using a REFLEX time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in the positive mode.

RESULTS

Heparin Binding Characteristics of Recombinant Fragments Containing Repeats III4–5–6 of Fn—Previous studies have identified a low affinity heparin/DNA-binding domain (Hep III) in the central region of Fn (1, 22–24). To further analyze the binding properties of this domain, we prepared recombinant fragments spanning Fn repeats III4–6, III4–5, III4, and III5, respectively (Fig. 1), and tested their ability to bind to heparin-Sepharose affinity matrices. As shown in Table I, all fragments bound to heparin but the conditions for elution from the matrix were different. The FN-III4–5 fragment showed the highest heparin-binding avidity and bound more strongly than the larger FN-III4–5–6 fragment. Full activity of FN-III4–5 apparently requires both repeats to be present since recombinant fragments containing single repeats (FN-III4 and FN-III5) bound to heparin with lower affinity (Table I).

The FN-III4–5 Fragment Mediates Cell Adhesion by Binding $\alpha\beta 1$ Integrin and CSPG, Role of Each Receptor Depends on the Stimulus Used to Activate $\alpha\beta 1$ —We have recently shown that the FN-III5 fragment mediates cell adhesion via interaction of the KLDAPT sequence (H2) with activated $\alpha 4$ integrins (27). To establish a possible correlation between the heparin binding activity of FN-III4–5 repeats and cell binding, we first tested whether the FN-III4–5 fragment also mediated cell adhesion. As shown in Fig. 2, resting Jurkat T cells did not bind to this fragment; however, upon incubation with 2 mM Mn^{2+} or the activating anti- $\beta 1$ mAb TS2/16, cells attached to this fragment in a dose dependent manner. This suggested the implication of a $\beta 1$ integrin as the receptor for FN-III4–5, possibly $\alpha\beta 1$ which is the receptor for FN-III5 (27). Since FN-III4–5 bound heparin with high avidity (Table I), we studied the contribution of both, $\alpha\beta 1$ integrin and cell surface PG for attachment to this fragment.

For these experiments, Jurkat cells were treated with either 2 mM Mn^{2+} or TS2/16 mAb and then incubated with anti- $\alpha 4$ mAb HP2/1, chondroitinase ABC, heparinase III, or the com-

FIG. 1. A, schematic drawing of the human Fn molecule showing the three types of internal homology units with type III repeats numbered. Shaded regions indicate the alternatively spliced segments (ED-B, ED-A, IIICS) only present in certain Fn subunits. The location of relevant sequences involved in cell adhesion via integrin or PG receptors, including the novel site (HBP/III5) reported here is indicated. The heparin-binding domains (Hep I, II, and III) of Fn are also shown. B, drawing and SDS-polyacrylamide gel electrophoresis analysis (15% acrylamide) of the recombinant fragments prepared and used in the present study. Numbers indicate the molecular mass (kDa) of known standards. (Modified from Moyano *et al.* (27)).

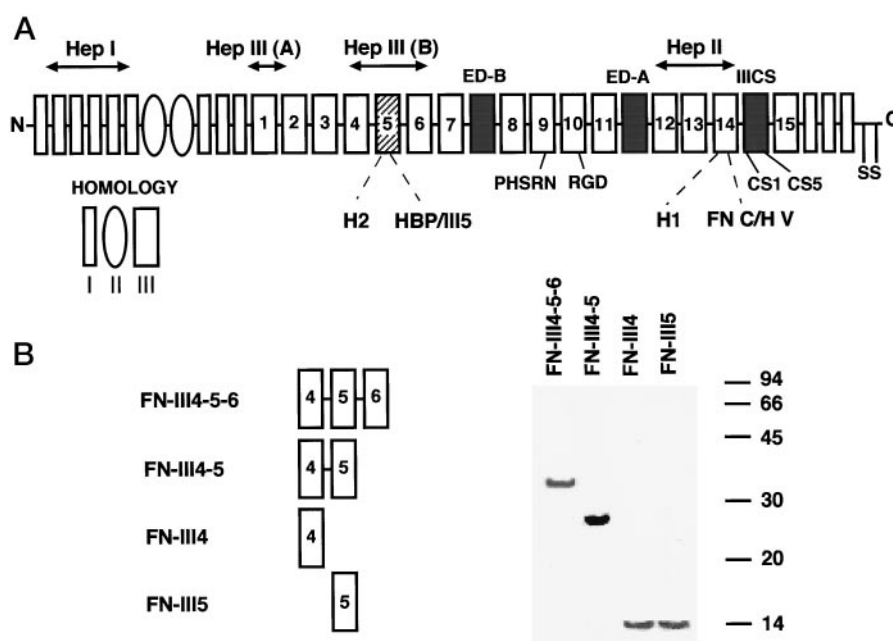


TABLE I
Heparin-Sepharose affinity chromatography of recombinant fragments derived from the Hep III domain of Fn

Fibronectin fragments	NaCl concentration required for elution
	nM
FN-III4-5-6	220
FN-III4-5	300
FN-III4	150
FN-III5	180

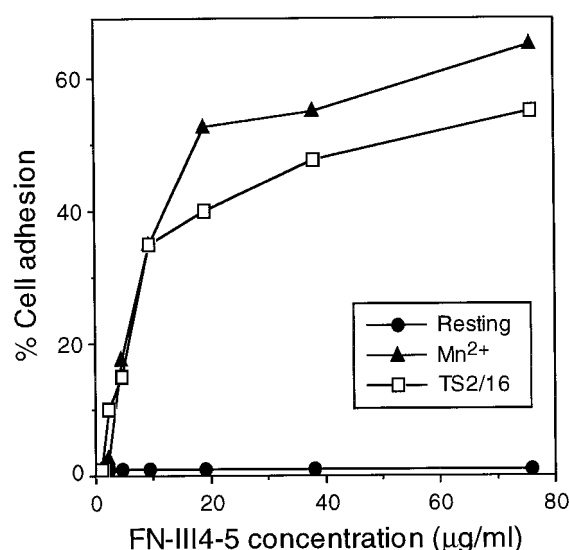


FIG. 2. Adhesion of Jurkat cells to increasing concentrations of FN-III4-5 fragment. Untreated cells (resting) or cells previously treated with Mn^{2+} or TS2/16 mAb were incubated for 3 h on wells coated with the indicated concentrations of FN-III4-5. Attached cells were quantitated as described under "Experimental Procedures." Values are the means of at least three different experiments with variability of <10%.

bination of mAb and enzymes prior to the attachment assay. As shown in Fig. 3, the contribution of $\alpha 4\beta 1$ and PG to cell adhesion to FN-III4-5 or FN-III5 fragments was different depending on whether the cells had been treated with Mn^{2+} or TS2/16. For cells incubated with Mn^{2+} , HP2/1 (1:5 dilution) had little effect on adhesion to either FN-III4-5 (27% inhibition) or FN-

III5 fragments (8% inhibition). However, treatment with chondroitinase ABC (1.0 units/ml) completely inhibited adhesion to both fragments (92 and 95% inhibition, respectively). Treatment with heparinase III (0.3 or 1 units/ml) or heparitinase (2 milliunits/ml, not shown) had no effect. Since chondroitinase ABC also degrades dermatan sulfate, we tested the effect of chondroitinase ACII which is specific for CS. At 1.0 units/ml, this enzyme inhibited adhesion to FN-III4-5 (91% \pm 8.3) and FN-III5 (93% \pm 2.1, mean of four experiments, data not shown), therefore indicating that the receptors for these fragments were CSPG.

Fig. 3 also shows that further dilutions of HP2/1 (1:50) or chondroitinase ABC (0.3 unit/ml), used individually, did not affect adhesion to either fragment (0–3% inhibition) of cells incubated with Mn^{2+} . Interestingly, when both reagents were combined at these concentrations, they inhibited cell adhesion to FN-III4-5 (65%) and FN-III5 (98%), thus suggesting a co-operation between both types of receptors. The residual binding to FN-III4-5 could not be inhibited under these conditions. As expected, the combination of HP2/1 (1:50) and heparinase III had no effect (Fig. 3).

Incubation of Jurkat cells with TS2/16 mAb resulted in a different pattern of adhesion to both fragments. As shown in Fig. 3, HP2/1 (1:5 dilution) efficiently inhibited (92%) adhesion to FN-III4-5 fragment and induced partial inhibition (62%) at 1:50 dilution. Chondroitinase ABC had a minor effect at 0.3 unit/ml (10% inhibition) and produced 60% inhibition at 1.0 unit/ml. Similar results were obtained with chondroitinase ACII (not shown). As observed for cells treated with Mn^{2+} , treatment with heparinase III (or heparitinase) did not affect adhesion. The combination of HP2/1 (1:50) and chondroitinase ABC (0.3 unit/ml) completely inhibited (99%) adhesion to the FN-III4-5 fragment while the combination of HP2/1 and heparinase III had no effect (Fig. 3).

The HP2/1 mAb also completely (100%, 1:5 dilution) or partially (45%, 1:50 dilution) inhibited adhesion to FN-III5 as we had previously reported (27). Chondroitinase ABC (1.0 unit/ml) had little effect in attachment to this fragment (22% inhibition) but when combined with 1:50 dilution of HP2/1 mAb, produced 100% inhibition (Fig. 3). Heparinase III, either alone or combined with HP2/1 (1:50 dilution), did not affect adhesion to the FN-III5 fragment (Fig. 3). To confirm that the heparinase enzymes were active in our assays, we tested their effect on cell

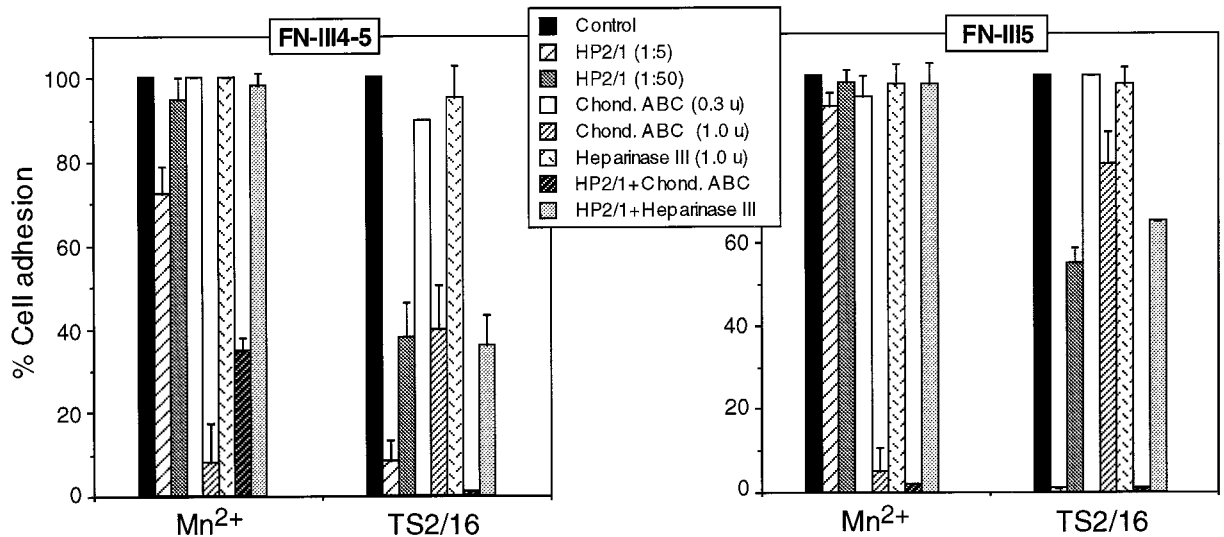


FIG. 3. **Effect of HP2/1 mAb and PG-degrading enzymes on cell adhesion to FN-III4-5 and FN-III5 fragments.** Mn^{2+} - or TS2/16-treated Jurkat cells were preincubated for 30 min with HP2/1 (*anti-α4*), chondroitinase ABC (*Chond.*), heparinase III, or mixtures of HP2/1 (1:50 dilution), and enzymes (0.3 unit/ml chondroitinase, 1 unit/ml heparinase III), and added to wells coated with FN-III4-5 or FN-III5 (38 μg/ml). After 3 h, attached cells were quantitated. Values represent percentage of control (no inhibitor) and are the means of at least five separate experiments. Standard deviation is indicated by bars.

adhesion to the H0 Fn fragment. H0 contains the Hep II domain and interacts with αβ1 integrin, CSPG, and HSPG (11, 28, 32). In results not shown, heparinase III (1 unit/ml) or heparitinase (2 milliunits/ml) had a minor effect when used alone but when combined with 1:100 dilution of HP2/1 mAb (which did not inhibit by itself) they produced >95% inhibition (mean of three experiments), indicating that the enzymes were active under the conditions used. Altogether these results indicate that adhesion to FN-III4-5 or FN-III5 fragments involves the cooperation of αβ1 and the glycosaminoglycan (GAG) chains of CSPG. CSPG play an important role when αβ1 is activated with Mn^{2+} while this integrin is the major receptor when activation is induced with TS2/16 mAb.

Identification of a 20-Residue Amino Acid Sequence in Fn III5 Repeat That Contains Heparin and Cell Binding Activities—The preceding results indicated that the FN-III4-5 fragment binds heparin and CSPG. To further define the specific sites(s) involved in these interactions, we compared amino acid sequences in repeats III4-III5 with previously characterized heparin or PG-binding sites in Fn. The sequence WTPPRAQITGY in III5 was highly homologous to WQPPRARITGY or FN-C/H V, previously shown to bind heparin and HSPG (12, 13). We therefore prepared several synthetic peptides spanning the WTPPRAQITGY sequence in III5 (Fig. 4) and tested their capacity to bind to heparin-Sepharose matrices and to mediate cell adhesion.

As shown in Fig. 5A, the synthetic peptides WTPPRAQITGY (HBP/III5-N11) and WTPPRAQITGYRLT (HBP/III5-N14) did not bind to heparin. However, the 20-residue peptide WTPPRAQITGYRLTVGLTRR (HBP/III5) as well as HBP/III5.sc, in which the sequence PPRAQIT was replaced by PQARPI, bound heparin very efficiently and >90% of the applied material was recovered in the 0.5 M NaCl fraction (Fig. 5A). Furthermore, peptide HBP/III5-C10, containing the C-terminal half of HBP/III5 (see Fig. 4), retained the heparin binding ability and approximately 41% of peptide eluted in the bound fraction (Fig. 5A). Substitution of the first arginine of C-10 by glutamine (peptide HBP/III5-C10.sc, Fig. 4) reduced the amount of bound peptide to 23% (Fig. 5A). The identity of peptides HBP/III5 and HBP/III5-C10 as the heparin-bound material was confirmed by amino acid analyses (not shown) as well as mass spectrometry analyses (Fig. 5B) of the 0.5 M NaCl eluted fractions. These

FN-C/H V:	WQPPRARITGY
HBP/III5-N11:	WTPPRAQITGY
HBP/III5-N14:	WTPPRAQITGYRLT
HBP/III5:	WTPPRAQITGYRLTVGLTRR
HBP/III5-C10:	YRLTVGLTRR
HBP/III5-C10.SC:	YQLTVGLTRR
HBP/III5.SC:	WTPQARPITGYRLTVGLTRR
H2:	KLDAPT

FIG. 4. **Synthetic peptides containing sequences from Fn type III5 repeat prepared and used in the present study.** Sequences are aligned with the previously identified peptide FN-C/H V (12). The H2 sequence was recently described as an active site in III5 (27). HBP, heparin-binding peptide; N or C refer to the NH₂- or COOH-terminal halves of HBP/III5, respectively; sc, scrambled.

results therefore show that the heparin-binding site contained in HBP/III5 resides in the C-terminal sequence YRLTVGLTRR and that all three arginine residues appear to be important for the interaction.

To determine whether the HBP/III5 peptide also mediated cell adhesion, resting, Mn^{2+} -treated, or TS2/16-treated Jurkat cells were added to wells containing increasing concentrations of peptide covalently coupled to KLH. As shown in Fig. 6, in all three cases Jurkat cells attached to HBP/III5 in a dose-dependent manner. Cells also attached similarly to the HBP/III5.sc peptide but not to the shorter HBP/III5-N11 or HBP/III5-N14, and attached only minimally (<20%) to HBP/III5-C10 and HBP/III5-C10.sc peptides (results not shown).

Cell adhesion to HBP/III5 was completely inhibited by chondroitinase ABC and ACII (1.0 unit/ml) and by soluble HBP/III5 peptide (0.5 mg/ml), regardless of the stimulus used for activation, while heparinase III (1 unit/ml), heparitinase (2 milliunits/ml, not shown), HP2/1 mAb (1:5 dilution) or 0.5 mg/ml of soluble H2 peptide (see Fig. 4) had no effect (Fig. 7). These results indicate that the full 20-residue sequence contained in HBP/III5 is necessary for an efficient adhesion and that adhe-

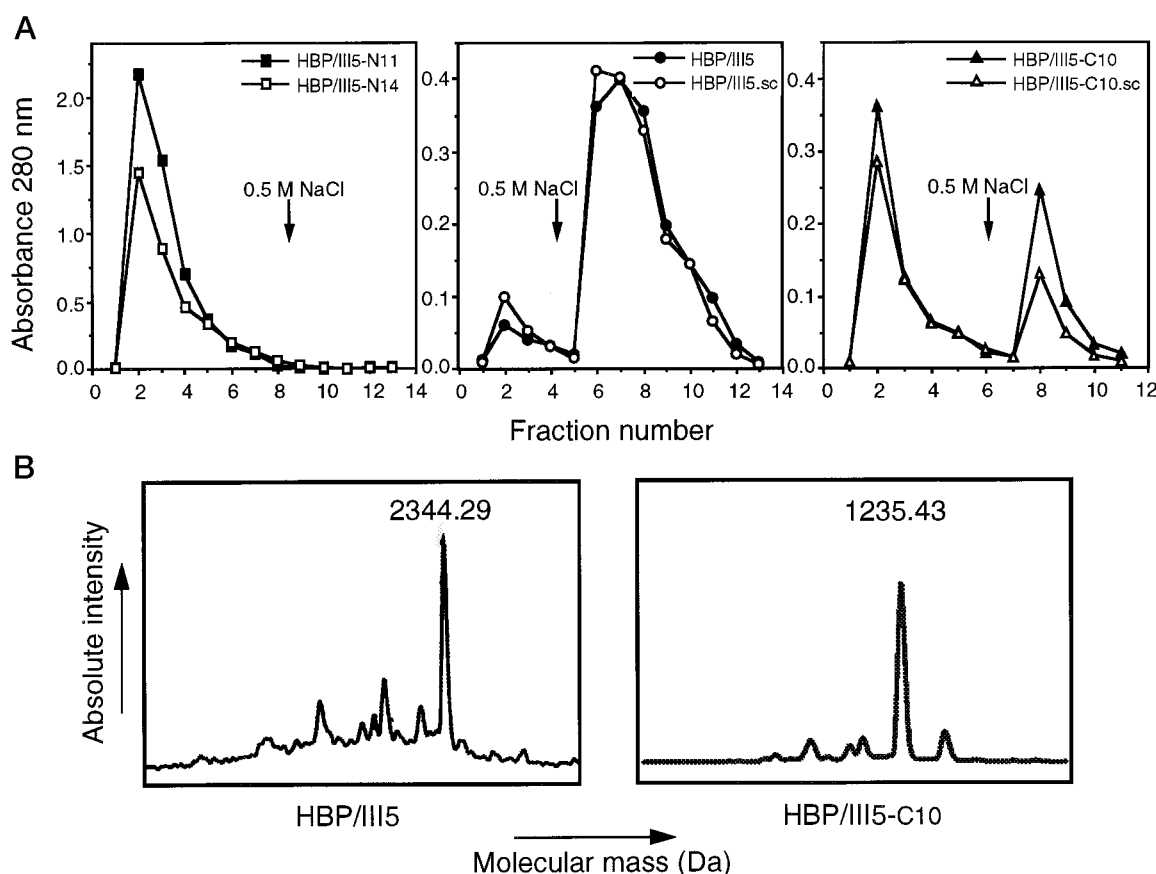


FIG. 5. Heparin binding properties of HBP/III5 synthetic peptide and smaller related constructs. **A**, elution profile from heparin-Sepharose affinity matrices of the indicated peptides. 0.4-ml fractions were collected and monitored by absorbance at 280 nm. **B**, mass spectrometry analysis of the 0.5 M NaCl eluted material from assays corresponding to peptides HBP/III5 and HBP/III5-C10.

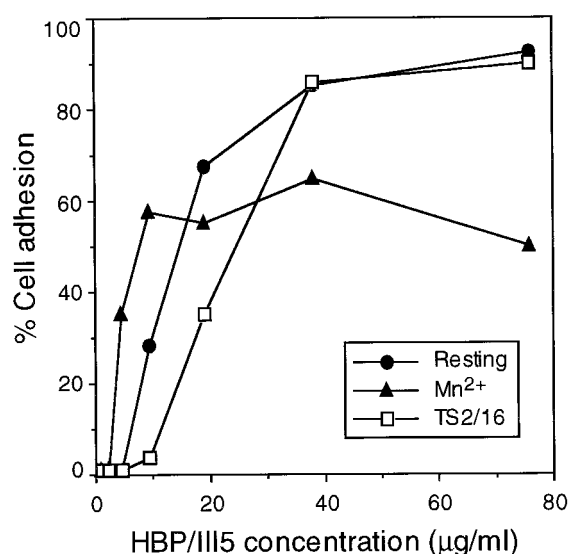


FIG. 6. Jurkat cell adhesion to increasing concentration of HBP/III5 peptide. Resting or treated cells (with Mn²⁺ or TS2/16) were incubated for 3 h on wells coated with the indicated concentrations of HBP/III5 coupled to KLH. Values are the mean of at least three experiments with variability of <10%.

sion to this sequence is exclusively mediated by CSPG.

Functional Role of HBP/III5 and H2 Sites in Cell Adhesion to FN-III5 and FN-III4-5 Fragments—The preceding results had identified a novel sequence in Fn III5 repeat, HBP/III5, which mediates cell adhesion via CSPG. Since III5 contains another sequence, KLDAPT (peptide H2) which binds activated

$\alpha 4$ integrins (27), we studied whether both sets of interactions acted in a coordinate manner to produce cell adhesion. The attachment of Jurkat cells to the previously described H2 peptide was first studied. As shown in Fig. 8, adhesion of Mn²⁺-treated cells to H2-KLH was completely inhibited by mAb HP2/1 or soluble H2 peptide while heparinase III had no effect. Interestingly, chondroitinase ABC and ACII also completely inhibited adhesion to H2 while soluble HBP/III5 peptide was a poor inhibitor (20% inhibition). For cells treated with TS2/16 mAb, adhesion to H2 was completely blocked by HP2/1 or soluble H2 peptide (Fig. 7) in agreement with our previous report (27), but not by soluble HBP/III5 (22% inhibition). Chondroitinases ABC and ACII in this case had little effect (15 and 8% inhibition, respectively) and heparinase III did not inhibit adhesion. These results suggest that for Mn²⁺-treated cells there is an interdependence of $\alpha 4\beta 1$ and CSPG receptors for recognition of the H2 sequence. For TS2/16-treated cells, however, adhesion is almost exclusively dependent on $\alpha 4\beta 1$ integrin.

To determine the contribution of the CSPG or $\alpha 4\beta 1$ -binding sites to cell attachment to FN-III4-5 or FN-III5 fragments, Mn²⁺- or TS2/16-treated cells were incubated with soluble HBP/III5 or H2 peptides prior to the adhesion assay. As shown in Fig. 9, adhesion to the FN-III5 fragment was clearly dependent on $\alpha 4\beta 1$ interaction with the H2 sequence since soluble H2 peptide almost completely blocked adhesion of Mn²⁺- or TS2/16-treated cells (90 and 93% inhibition, respectively). Soluble HBP/III5 peptide had a minor effect (8–15% inhibition) although in combination with H2 increased the inhibition to 100% in both cases.

In the case of FN-III4-5, the HBP/III5 peptide alone produced a 13% inhibition regardless of the activation stimulus

FIG. 7. Inhibition of cell adhesion to HBP/III5-KLH by chondroitinase ABC and chondroitinase ACII. Resting or treated (with Mn^{2+} or TS2/16) Jurkat cells were preincubated for 30 min with HP2/1 (1:5 dilution), chondroitinase ABC or ACII (1.0 unit/ml), heparinase III (1 unit/ml), or soluble HBP/III5 or H2 peptides (0.5 mg/ml each), and added to wells previously coated with HBP/III5-KLH (19 μ g/ml). Attached cells were quantitated after 3 h. Values are expressed as percentage of control (no inhibitor) and are the mean of five different experiments.

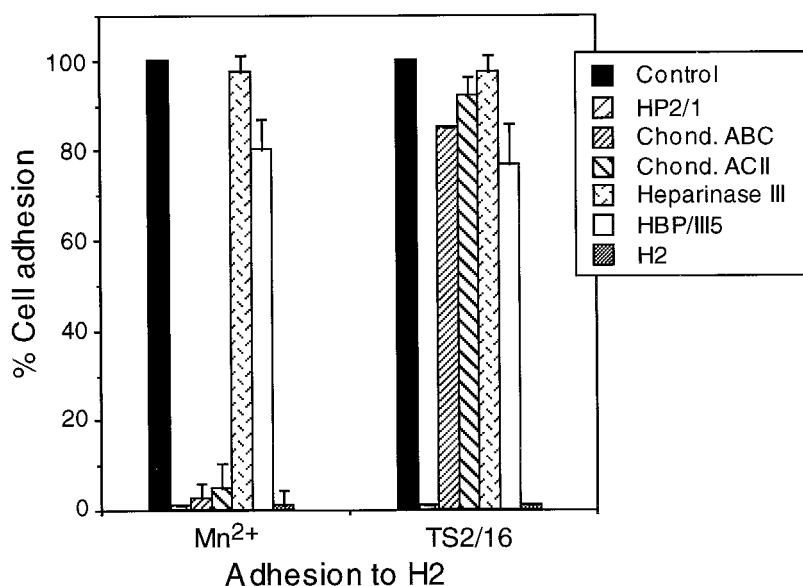
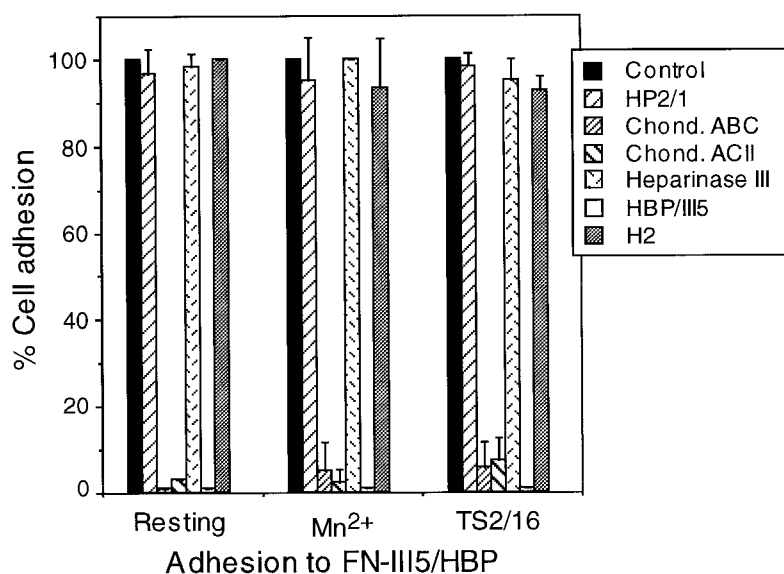
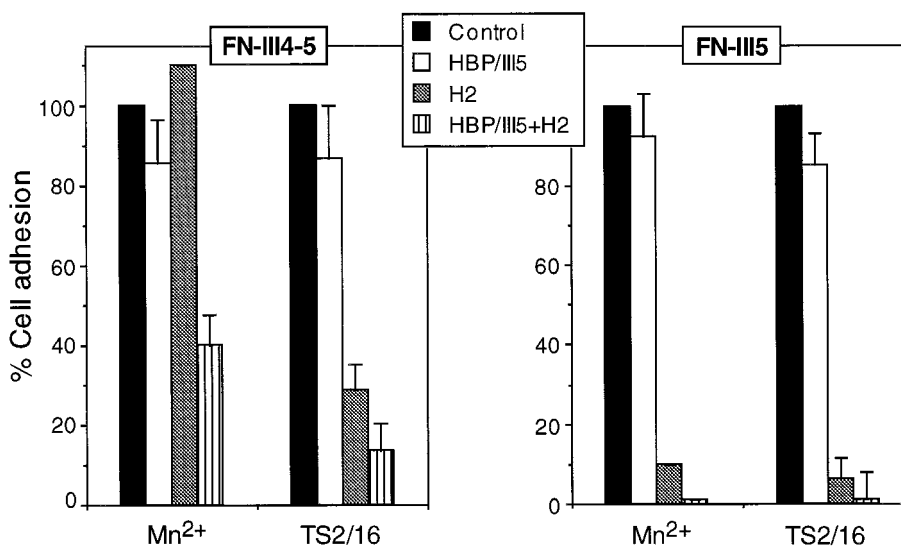


FIG. 8. Effect of HP2/1, PG-degrading enzymes, and soluble peptides on cell adhesion to the H2 peptide. Mn^{2+} - or TS2/16-treated cells were preincubated for 30 min with the indicated reagents and added to wells coated with H2-KLH (19 μ g/ml). Attached cells were quantitated after 3 h. Values are expressed as percentage of control (no inhibitor) and are the mean of five different experiments.

FIG. 9. Effect of soluble peptides on cell adhesion to FN-III4-5 and FN-III5 fragments. Mn^{2+} - or TS2/16-treated Jurkat cells were preincubated for 30 min with HBP/III5 or H2 peptides (0.5 mg/ml) and added to wells coated with FN-III4-5 or FN-III5 (38 μ g/ml). After 3 h, attached cells were quantitated. Values are expressed as percentage of control (no peptide) and are the mean of five different experiments.



used; the H2 peptide did not inhibit adhesion (in fact it increased it slightly) of Mn^{2+} -treated cells but produced 71% inhibition on TS2/16-activated cells (Fig. 9). Interestingly, the

combination of both peptides effectively inhibited (60%) the adhesion of Mn^{2+} -treated cells and increased the inhibitory effect of H2 for TS2/16-treated cells to 86% (Fig. 9). The resid-

ual adhesion observed in both conditions could not be inhibited by these reagents. These results indicate that both sites H2 and HBP/III5 participate in adhesion to the FN-III4–5 fragment when $\alpha 4 \beta 1$ is activated with Mn^{2+} , but H2 is prevalent when the integrin is activated with TS2/16 mAb.

DISCUSSION

The function of the Hep III domain of Fn has been largely unknown mainly because proteolytic fragments corresponding to this region do not bind heparin (or DNA) at physiological salt concentrations (22–24). We have recently shown that a recombinant fragment containing repeat III5, within the Hep III domain induced cell adhesion via interaction of the H2 site (KLDAPT) with activated $\alpha 4$ integrins, thus establishing a novel function for this region of Fn (27).

In this report we have further characterized the heparin and cell binding activities of this domain. We show that a recombinant fragment containing repeats III4–III5 (FN-III4–5) bound heparin at physiological NaCl concentrations and with higher avidity than the larger fragment FN-III4–6 (see Table I). One possible explanation for this is that the site(s) contained in repeats III4–III5 is partially cryptic and removal of repeat III6 fully exposes it and increases the avidity of the interaction. Fn contains several cryptic sites including those involved in self-association (33) and chemotaxis (34). A recent study has shown that physical tension (such as that produced by tissue injury) exposes cryptic sites in the Fn molecule (35), thus confirming the biological relevance of these “latent” regions. These regions may also become functional upon proteolytic degradation of Fn at sites of tissue damage.

The FN-III4–5 fragment also induced cell adhesion and this involved the cooperation of $\alpha 4 \beta 1$ integrin and the GAG chains of CSPG. Early studies had shown that HSPG binding to fragments containing the Hep II domain was necessary for focal adhesion and stress fiber formation of fibroblasts attached to the central cell-binding domain of Fn (36). Other reports have demonstrated that melanoma cell adhesion to fragments or peptides from the Hep II domain involves the cooperation of CSPG and $\alpha 4 \beta 1$ integrin (11, 15–18), that CSPG can modulate the function of $\alpha 4 \beta 1$ (11), and that this regulation may be exerted by direct interaction of CSPG with $\alpha 4$ via a newly identified functional GAG-CS-binding site in this integrin subunit (17).

Our present results on the Hep III domain are in agreement with these previous studies on the COOH-terminal region of Fn and show important differences between cell adhesion to the Hep II and Hep III Fn domains. First, adhesion to FN-III4–5 and FN-III5 fragments required previous activation of $\alpha 4 \beta 1$ with Mn^{2+} or TS2/16. Second and most importantly, the role of $\alpha 4 \beta 1$ and CSPG was clearly dependent on whether $\alpha 4 \beta 1$ had been activated with Mn^{2+} or TS2/16 mAb. We and others have previously shown that these two agents induce high affinity forms in $\alpha 4 \beta 1$ and it was generally assumed that both lead to similar activation states (19, 20, 37). However, careful examination of these previous reports reveals subtle differences with respect to the effects of Mn^{2+} and TS2/16. For example, Masumoto and Hemler (37) showed that in cells with constitutively low $\alpha 4 \beta 1$ activity, Mn^{2+} stimulated adhesion to CS-1 and VCAM-1, whereas TS2/16 only induced adhesion to VCAM-1. We have also reported (20) that TS2/16 was 2–3-fold more effective than Mn^{2+} in inducing recognition of the Hep II domain of Fn by monocytic cells. Likewise, TS2/16 but not Mn^{2+} enhanced the constitutive adhesion of these cells to a Fn fragment containing CS-1 (20).

In this report we clearly demonstrate functional differences between activation of $\alpha 4 \beta 1$ with Mn^{2+} or TS2/16, which results in a higher dependence of GAG-CSPG in the case of Mn^{2+}

treatment. The effect of Mn^{2+} was not due to a charge neutralization of the sulfated chains of GAG since other divalent cations (Ca^{2+} , Mg^{2+} , resting conditions) did not induce cell adhesion. Assuming that $\alpha 4 \beta 1$ and CSPG form a complex at the cell surface, we can postulate that activation with Mn^{2+} results in a partially active $\alpha 4 \beta 1$ unable to support adhesion by itself after disruption of the complex with chondroitinase ABC. In contrast, TS2/16 would lock the integrin in an active conformation which would no longer require cooperation by CSPG. In support of this, chondroitinase ABC and chondroitinase ACII completely inhibited adhesion to the synthetic peptide H2 (which does not bind PG) when $\alpha 4 \beta 1$ was activated with Mn^{2+} , but had a minor effect on TS2/16 activation. These results differ from previous findings on melanoma cells where activation of $\alpha 4 \beta 1$ with either reagent reverted the effect of chondroitinase ABC (17). An explanation for this could be a different constitutive activity of $\alpha 4 \beta 1$ in melanoma and lymphoid cells (our study) thus implying a different regulation by CSPG.

We have also identified a novel amino acid sequence in Fn repeat III5, WTPPRAQITGYRLTVGLTRR (named HBP/III5), which binds heparin and mediates cell adhesion via CSPG. Although the NH_2 -terminal half of this sequence is highly homologous to the previously described WQPPRARITGY or FN-C/H V located in repeat III14 (12), HBP/III5 required all 20 amino acid residues for full activity and this was partially retained in the last 10 residues but not in the NH_2 -terminal portion of the peptide. This indicates that the sequence requirements are different for HBP/III5 and FN-C/H V and that the three arginine residues of the COOH-terminal portion seem to be crucial for activity of the former. The nature of the GAG chains that interact with FN-C/H V and HBP/III5 may also be different. In our study, HBP/III5 as well as fragments FN-III4–5 and FN-III5 clearly bound CS but not HSPG. Although this conclusion is based on the lack of effect of heparinase III or heparitinase in the adhesion assays, we have confirmed that these enzymes were active under identical conditions when tested on a substrate previously known to interact with HSPG (32, 36). Peptide FN-C/H V, however, was originally shown to bind only HSPG (13) although phorbol 12-myristate 13-acetate-treated U937 monocytic cells apparently bind this peptide through both types of PG (14). It is therefore possible that the differential use of CS or HS GAG chains for interactions with Fn depends on the nature of the ligand and/or on the cell type of study.

Based on the present results we can establish that repeat III5 contains two closely located active sites, the previously described H2 which binds activated $\alpha 4$ integrin and HBP/III5 which binds CSPG. Both sites cooperate in mediating cell adhesion to the Hep III domain. Moreover, we have consistently observed that adhesion to FN-III4–5 (but not to FN-III5) could not be completely inhibited neither with the combination of chondroitinase ABC and HP2/1 mAb nor with the mixture of H2 and HBP/III5 peptides, suggesting that additional active sites may exist in this fragment. These sites could be located in repeat III4, however, a recombinant fragment containing only this repeat (FN-III4) did not mediate adhesion of Jurkat cells (results not shown). This suggests that additional sites may require the entire III4–III5 region for activity, a fact that is essential for the high affinity binding of this domain to heparin (see Table I).

The physiological significance of the Hep III domain is beginning to be revealed. Besides our previous demonstration of the cell binding activity of repeat III5 and the results presented here, other authors have shown that: 1) the region encompassing repeats III1–III7 may regulate Fn matrix formation (38); 2) a mAb recognizing an epitope in repeat III5 inhibited fibro-

blast-mediated collagen gel contraction (39), suggesting a role for this region in interactions with collagen. Although further work is necessary to completely understand the function of this domain, it is possible that repeats III4-III5 are involved in the process of Fn matrix formation by interacting with cells as well as with other macromolecules. In this regard, it was recently shown that repeats III12-14 in the Hep II domain participate in Fn polymerization (40). The III4-III5 region may also constitute an important cell attachment domain for activated leukocytes at inflammatory sites and injured tissues, where proteolytic degradation or conformational changes of Fn take place physiologically.

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Cooperative Role for Activated $\alpha 4 \beta 1$ Integrin and Chondroitin Sulfate Proteoglycans in Cell Adhesion to the Heparin III Domain of Fibronectin: IDENTIFICATION OF A NOVEL HEPARIN AND CELL BINDING SEQUENCE IN REPEAT III5

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