Conformational Transitions in the Cell Binding Domain of Fibronectin†

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ABSTRACT: Plasma fibronectin readily changes shape in response to environmental conditions which may, in turn, lead to differential expression of its multiple functional sites. To test this possibility, the expression of two of the type III modules within cell binding domain of fibronectin was assessed with monoclonal antibodies (mAb). Utilizing proteolytic and recombinant fragments of plasma fibronectin, the epitopes recognized by mAbIII-9 and mAbIII-10 were localized to the ninth and tenth (RGD-containing) type III repeats of fibronectin, respectively. Both mAb inhibited the adhesion of platelets to immobilized fibronectin, suggesting that the recognized epitopes resided in close spatial proximity to the cell binding sites. Radioimmunoassay and Scatchard analyses showed that, in solution, each dimeric fibronectin molecule bound two mAbIII-9 but only one mAbIII-10 molecule (ionic strength 0.15, pH 7.4). The binding of a single mAbIII-10 per fibronectin molecule was verified by electron microscopy. Heparin, heparan sulfate, gangliosides (but not chondroitin sulfates A and B and hyaluronic acid), and self-association increased the apparent affinity of mAbIII-10 for soluble fibronectin. Adsorption of fibronectin onto a polystyrene surface resulted in the appearance of an additional binding site for mAbIII-10. MAbIII-9 binding also was altered by fibronectin immobilization. These results suggest that the deposition of fibronectin and its interaction with components of the extracellular matrix can modulate the expression of the cell binding domains including the RGDS-containing type III repeat. Exposure of the second tenth type III repeat within the fibronectin dimer, as a result of unfolding on a surface, could contribute to the enhanced adhesiveness of adsorbed fibronectin.

Plasma fibronectin (Fn)[1] mediates the adhesive reactions of cells involved in biological processes ranging from hemostasis and wound healing to phagocytosis (Reviewed in Mosher, 1988; Ruoslahti, 1988; Hynes, 1990). The molecule is primarily dimeric, consisting of two very similar but not identical subunits of 250 and 235 kDa. Each subunit is composed of several domains which exhibit distinct functions such as affinity for specific ligands including fibrin, heparin, collagen, DNA and for cell-surface receptors. These domains, in turn, are assembled from three types of structural modules which occur in multiple repeats.

Intramolecular associations between nonadjacent domains within the same subunit and between domains of the two constituent chains (Homandberg & Erickson, 1986; Robinson & Hermans, 1984; Hörmann & Richter, 1986; Litvinovich et al., 1991; Matsuka et al., 1994) result in folding of Fn into a complex structure. Moreover, Fn is highly flexible and its shape can be markedly affected by solution conditions and by deposition onto surfaces. High ionic strength, pH extremes, and glycerol can cause reversible transitions of Fn from a "compact" shape to an "expanded" conformation without alteration in the global fold of the molecule as evaluated by physicochemical methods (Alexander et al., 1979; Williams et al., 1982; Rocco et al., 1983; Benecky et al., 1991; Lai et al., 1993). On the other hand, deposition of Fn on surfaces can result in irreversible unfolding into an "extended" conformer. The flexibility of Fn is evidenced by its variability in shape as assessed by electron microscopy: plasma Fn has ranged from a compact particle to an elongated, rod-like structure (Erickson & Carrel, 1983; Tooney et al., 1983; Engel et al., 1981; Price et al., 1982).

Conformational flexibility has the potential to influence the biological properties of Fn. Consistent with this possibility, certain activities of Fn are latent in the intact molecule and become fully expressed in its proteolytic fragments (Czop et al., 1981; Norris et al., 1982; DePetro et al., 1981; Sekiguchi et al., 1983; Homandberg et al., 1985; Muir & Manthorpe, 1992). For example, 29 and 40 kDa heparin-binding proteolytic fragments of Fn are potent inhibitors of endothelial growth in vitro, while native Fn is not (Homandberg et al., 1985). Cell binding, critical to so many of the functions of fibronectin, also may be regulated by Fn conformation. Soluble plasma Fn binds poorly to many cell types in suspension (Pearlstein, 1978; Grinnell et al., 1982; Bevilacqua et al., 1981; Rennard et al., 1981), but

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[1] Abbreviations: Fn, human plasma fibronectin; mAb, monoclonal antibody; PBS, (phosphate-buffered saline), 0.01 M sodium phosphate buffer, pH 7.4, and 0.15 M NaCl; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; μ, ionic strength.

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after deposition onto a suitable substrate, its cell binding avidity is enhanced (Pearlstein, 1978; Grinnell, 1980; Rennard et al., 1981).

In the present study, we have assessed the availability of cell recognition modules in the different conformational states of plasma Fn using site-directed monoclonal antibodies. One antibody (mAbIII-10) is directed against the 10th type III homology segment which contains an RGDS sequence responsible for mediating the adhesive activity of Fn, dependent upon its interaction with several integrin receptors (Ruoslahti, 1991). The second antibody (mAbIII-9) recognizes an epitope residing in the 9th type III homology segment. This region contains a cell binding site for the platelet integrin $\alpha_{IIb}\beta_3$ (Bowditch et al., 1991, 1994). The results show that access to these regions is greatly and differentially influenced by the conformational states of Fn.

EXPERIMENTAL PROCEDURES

Materials. Gelatin−agarose, protein A−agarose, Sepharose 2B CL, and Sephacryl S-200 and S-300 were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). Chymotrypsin, heparin, heparan sulfate, chondroitin sulfate A and B (dermatan sulfate), hyaluronic acid in monomeric and polymeric forms, gangliosides G_{D1A}, G_{M1}, G_{T1b}, and G_{M3}, BSA, and prostaglandin E_1 were from Sigma Chemical Co., St. Louis, MO. Analytical quantities of 110 kDa chymotryptic fragment of Fn were purchased from Telios Pharmaceuticals, La Jolla, CA. Goat anti-mouse IgG, conjugated with alkaline phosphatase, and bis(sulfosuccinimidyl) suberate were from Pierce Chemical Co., Rockford, IL. *N*-Hydroxysuccinimide biotin ester and avidin, conjugated to alkaline phosphatase, were from Calbiochem. The reagents for radioimmunoassay (normal mouse serum and goat anti-mouse serum) were purchased from Pel-Freez, Rogers, AK. Polystyrene microtiter plates were Immulon 2 from Dynatech Laboratories, Inc., Chantilly, VA. Immobilon-P membranes were purchased from Millipore Corp., Bedford, MA, and peroxidase-conjugated second antibody was from Bio-Rad Laboratories, Richmond, CA.

Isolation of Human Plasma Fn and Its Derivatives. Fn was isolated from fresh blood, anticoagulated with acid/citrate/dextrose, by gelatin−agarose affinity chromatography using 1 M arginine for elution (Vuento & Vaheri, 1979). After extensive dialysis against PBS, Fn was stored in aliquots at −70 °C. Upon thawing, Fn was maintained at 4 °C and used within 3 days. When analyzed by SDS−PAGE on 5% acrylamide gels under nonreducing conditions, about 90% of the Coomassie blue staining protein migrated as the Fn dimer.

The 110 kDa fragment was prepared by digestion of Fn (3.7 mg/mL) in PBS with chymotrypsin at an enzyme: substrate ratio (w/w) of 1:100. Proteolysis was terminated after 2 h at 22 °C by addition of 1 mM phenylmethanesulfonyl fluoride, and the digest was subjected to gel filtration on Sephacryl S-200, equilibrated with PBS. Fractions containing a fragment of 110 kDa were pooled, and its concentration was determined using an extinction coefficient of 1.0 (Homandberg & Erickson, 1986). The proteolytic fragments of Fn used to map the epitope of mAbIII-4 were gifts from Dr. S. Litvinovich, American Red Cross, Rockville, MD. Recombinant fragments spanning sequences of 9th and 10th type III homology segments of Fn were expressed in *Escherichia coli* as fusion proteins with maltose binding protein. The preparation and characterization of these fragments have been previously described (Bowditch et al., 1991, 1994). Fn and the monoclonal antibodies were radiolabeled with ^{125}I by a modified chloramine-T procedure as previously described (Plow & Ginsberg, 1981).

Production of mAb. MAb were produced by immunizing mice with tryptic digests of Fn. Cell fusions to obtain hybridomas were performed as previously described (Shadle et al., 1984). Three mAb (clones 5A11, 8C5, 1B7) were selected for study on the basis of their reactivity in ELISA with the 110 kDa cell-binding (chymotryptic) fragment of Fn. A fourth mAb, clone 8A11, which did not react with this fragment, was selected as a control. All four Fn mAb were of the IgG_1 subclass and had κ light chains. They were purified by protein A−agarose affinity chromatography and were homogeneous as judged by SDS−PAGE and electron microscopy. The four mAb did not compete with each other for binding to immobilized Fn. Potential cross-competition was assessed in an ELISA format in which each mAb was tested for its capacity to inhibit the binding of each biotinylated mAb to immobilized Fn. For competition experiments, 100 μL aliquots of mixtures containing varying concentrations of unlabeled antibodies and biotinylated antibody (3 μg/mL) were added to the wells coated with 2 μg/mL Fn and incubated 2 h at 37 °C. After 4 washes with PBS + 0.05% Tween 20, avidin conjugated with alkaline phosphatase was added and incubated for 1 h. After washing, mAb binding was detected by reaction with *p*-nitrophenyl phosphate. Antibodies were biotinylated with *N*-hydroxysuccinimide biotin ester as described (Shattil et al., 1987).

mAb Binding to Soluble Fn (RIA). A double antibody radioimmunoassay was employed to determine the affinity and stoichiometry of mAb binding to soluble plasma Fn in the presence of different effector molecules. ^{125}I-Fn, from 0.5 to 50 nM, and unlabeled mAb, 1 nM, in a total volume of 0.3 mL of PBS, containing 3% BSA and 2% normal mouse serum (added as a source of immunoglobulin to assist in immunoprecipitation) (RIA buffer), were incubated for 4 h at 22 °C. This condition was at apparent equilibrium, as the percent of the radiolabeled Fn bound did not change with further incubation. Goat anti-mouse antiserum was then added, and after 2 h at 22 °C, the tubes were centrifuged. The pellet was resuspended by vortexing in 0.5 mL of PBS, collected again by centrifugation, and counted in a γ-counter. The amount of ^{125}I-Fn bound was calculated from its specific activity. In some experiments, the normal mouse serum used in RIA was depleted of Fn by affinity chromatography on gelatin agarose, concentrated to the initial serum volume, and added to RIA buffer. The same results were obtained as with nondepleted serum. The binding constants of Fn-mAb interactions were determined by Scatchard analyses (Scatchard, 1949) using the EBDA Program (McPherson, 1987, Elsevier-Biosoft, Cambridge, U.K.).

mAb Binding to Immobilized Fn. Fn, at a concentration of 2 μg/mL (100 μL) in PBS, was coated on Immulon 2 Removawell strips overnight at 4 °C. After post-coating with 1% BSA, various concentrations of ^{125}I-mAb, in 0.1 mL of PBS + 0.1% BSA, were added to the wells for 2 h at 22 °C. After washing with PBS + 0.05% Tween, bound radioactivity was measured and the amount of bound mAb was calculated, correcting for nonspecific binding to BSA-coated

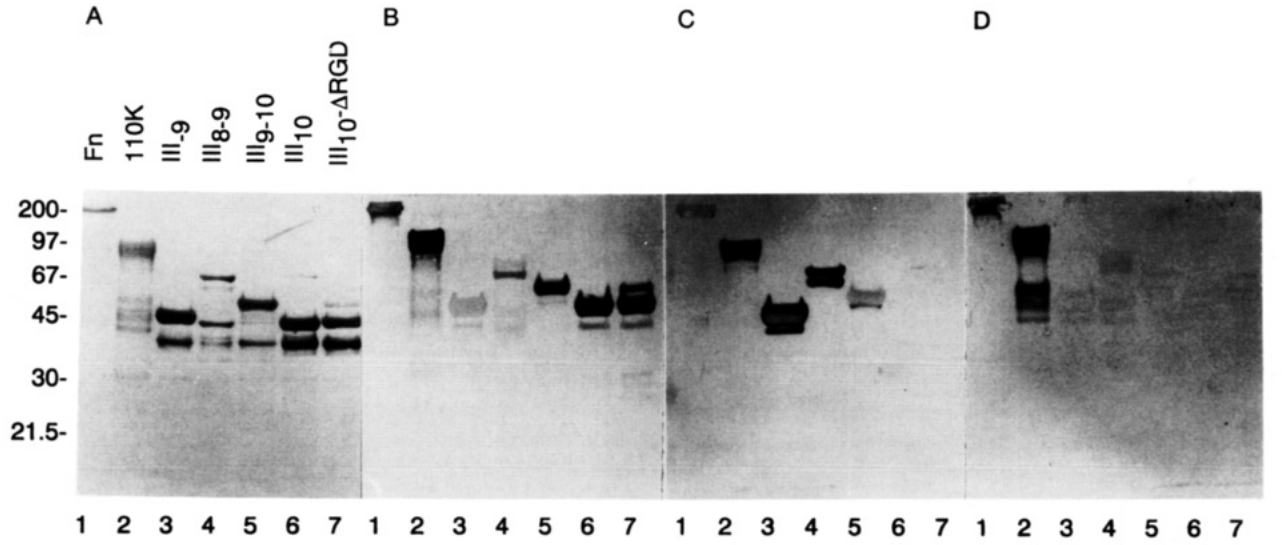

FIGURE 1: Mapping the epitopes for mAbIII-9, mAbIII-10, and mAbIII-4. Fn reduced with β-mercaptoethanol (lane 1), chymotryptic 110K fragment (lane 2), and recombinant fragments spanning sequences of 9th repeat (III_9), 8 to 9th repeat (III_{8-9}), 9 to 10th repeat (III_{9-10}), 10th repeat (III_{10}), or 10th repeat with deleted RGDS ($III_{10\text{-}RGDS}$) (lanes 3−7), respectively, were electrophoresed on 10% SDS−PAGE. Proteins were transferred on Immobilon P membrane and stained for protein (A) or probed with mAbIII-10 (B), mAbIII-9 (C), and mAbIII-4 (D). Recombinant fragments are fusion proteins with maltose binding protein. Lower molecular weight bands in each recombinant preparation are proteolytic fragments. Positions of M_r markers are shown.

wells. To determine the amount of Fn bound to the wells, in each experiment, a set of wells were coated with 2 μg/mL ^{125}I-Fn (100 μL), and the wells were processed as described above. The amount of bound Fn was typically about 35% of input Fn.

Competitive Inhibition ELISA. This assay was used as a semiquantitative approach to assess expression of epitopes in soluble Fn under selected conditions. Briefly, the selected mAb was diluted so as to be the limiting component in the assay and then mixed with Fn. Aliquots (100 μL) of these mixtures containing 10 μg/mL Fn and 1 μg/mL mAb (final concentrations) were immediately added to duplicate wells of Immulon 2 microtiter plates which had been coated with Fn (100 μL/well at 2 μg/mL) and post-coated with 1% BSA. After incubation for 1.5 h at 22 °C, the plates were washed, and goat anti-mouse IgG, conjugated to alkaline phosphatase, was added. MAb binding was detected by reaction with *p*-nitrophenyl phosphate.

Cell Adhesion Assay. Fresh aspirin-free human blood, anticoagulated with acid/citrate/dextrose and drawn into 2.8 μM prostaglandin E_1 and 1 mM theophylline, was used to isolate platelets. The platelets were labeled with $Na_2{}^{51}CrO_4$ (1 mCi for 30 min) in platelet-rich plasma and isolated by gel filtration on Sepharose 2B CL in divalent cation-free Tyrode buffer, pH 7.2, in the presence of 20 ng/mL prostaglandin E_1. Platelets were mixed with different concentrations of mAbIII-10 and mAbIII-9 in Hanks balanced salt solution + 0.1% BSA in the presence of 2.5 mM each Ca^{2+}, Mn^{2+}, and Mg^{2+} and incubated for 30 min at 22 °C. Then 10^7 cells in triplicates were added to individual wells of Immulon 2 microtiter plates, coated with Fn (100 μL at 10 μg/mL), and post-coated with 1% BSA, for 2 h at 37 °C. Nonadherent cells were removed by washing with Hanks + 0.1% BSA, attached cells were solubilized with 2% SDS for 30 min, and bound ^{51}Cr was quantitated in a β-counter.

Electron Microscopy. Mixtures of Fn and mAbs, at molar ratio 1:1 and 1:2, were incubated in PBS for 4 h at 22 °C. Before spraying on mica, the mixtures were diluted 20-fold in 0.1 M ammonium formate buffer, pH 7.4, containing 30% glycerol to give a final protein concentration of 25 μg/mL. The samples were dried and rotary shadowed with tungsten at an angle of 9°, followed by carbon coating in a vacuum evaporator (Denton Vacuum Co., Cherry Hill, NJ) (Veklich et al., 1993). The replicas were floated onto the surface of distilled water, picked up onto copper grids, and examined in a Phillips 400 electron microscope (Phillips Electronic Instruments Co., Mahwah, NJ), operating at 80 kV and at a magnification of 60000×.

Analytical Procedures. SDS−PAGE was performed under nonreducing or reducing conditions on 5-10% acrylamide gels in a Laemmli buffer system (Laemmli, 1970). For Western blots, the separated proteins were transferred onto Immobilon P membranes, and the filters were incubated with selected mAb (10 μg/mL). Bound mAb were detected by reaction with a peroxidase-conjugated second antibody, followed by addition of the 4-chloro-1-naphthol substrates.

RESULTS

Epitope Localization. Four mAb elicited to a tryptic digest of Fn were utilized in this study. Three of these mAb reacted with the 110 kDa cell-binding chymotryptic fragment of Fn (Figure 1), and their epitopes were localized. Epitope mapping was carried out by Western blotting using recombinant fragments spanning sequences of 9th and 10th type III homology segments of Fn. MabIII-10 (clone 5A11) and mAbIII-9 (clone 8C5) recognized epitopes residing in 10th and 9th type III modules of Fn, respectively (Figure 1B,C). Deletion of the RGDS sequence from the fragment containing the sequence of 10th repeat did not affect the reactivity of mAbIII-10. The placement of the recognition boundaries of mAbIII-4 (clone 1B7) was based upon its interaction with defined Fn proteolytic fragments by Western blotting. This mAb reacted with a 54 kDa fragment, containing the 2nd to 6th type III modules, but not with a 21 kDa fragment, containing the 5+6th type III modules or with a 22 kDa fragment containing the 2+3rd type III modules (not shown).

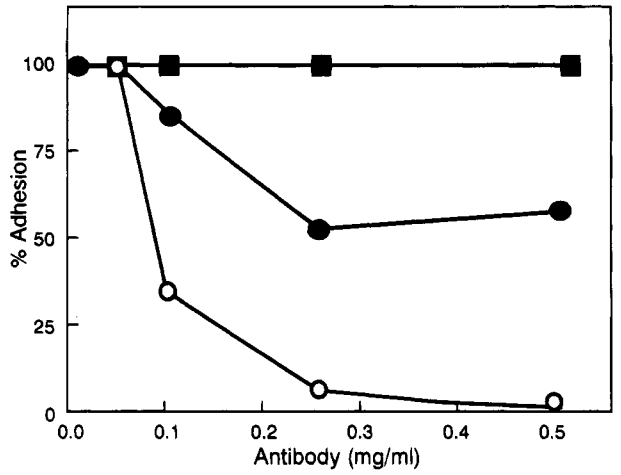

FIGURE 2: Inhibition of cell adhesion to Fn by mAbIII-10 and mAbIII-9. ^{51}Cr-labeled platelets were incubated with varying concentrations of mAbIII-10 (○), mAbIII-9 (●), or mAbIII-4 (■) for 30 min at 22 °C. The cells were then allowed to attach to a Fn-coated surface for 2 h. At the end of this time, the nonadherent platelets were removed by washing, the adherent cells were solubilized in SDS, and bound ^{51}Cr was quantitated. Values are expressed as the percent of adhesion in the absence of mAb. The experiment shown is representative of four separate determinations.

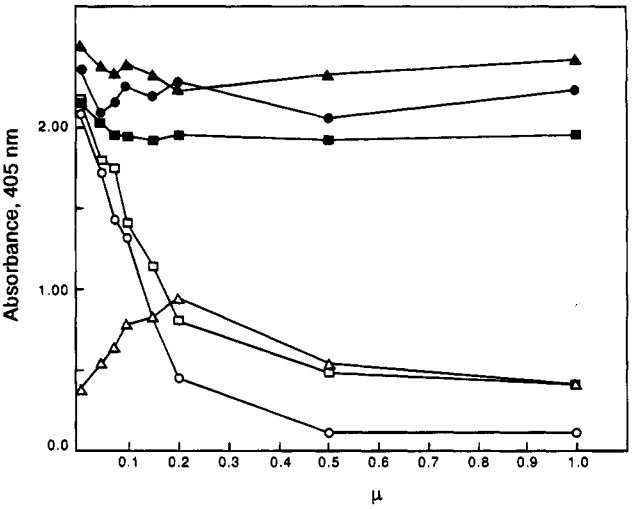

FIGURE 3: Effect of ionic strength on the expression of epitopes in the 9th and 10th type III homology segments in soluble Fn. Soluble Fn, at 10 μg/mL, was used in an ELISA as competitor of mAbIII-10 (○), mAbIII-9 (□), and mAbIII-4 (△) binding to Fn coated onto microtiter wells. Fn and each mAb were diluted with 0.01 M phosphate buffer, pH 7.4, containing NaCl to obtain the selected μ, mixed, and added to the wells. The closed symbols (● = mAbIII-10; ■ = mAbIII-9; ▲ = mAbIII-4) indicate binding of the mAb to immobilized Fn in the absence of soluble Fn. Representative of three experiments is shown.

The recognition specificity for mAb 8A11 was not further defined.

Functional Properties of mAb. The 9th and 10th type III homology segments of Fn contain cell binding sites for specific integrin receptors. Accordingly, we sought to determine whether the mAb to these segments affected the adhesion of cells to Fn. Platelets were utilized in this analysis as these cells express integrins that recognize both the 9th and the 10th ($\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$) repeats. As shown in Figure 2, both mAb inhibited platelet adhesion to Fn in a concentration-dependent manner. MAbIII-10 completely abolished adhesion at a concentration of 500 μg/mL, whereas mAbIII-9 partially blocked platelet attachment. The control mAb to the 4th type III repeat had no effect on platelet adhesion. These data indicate that the epitopes recognized by mAbIII-9 and mAbIII-10 reside in close spatial proximity to the cell binding sites in Fn and further suggest that these mAb could be effective reporters of changes in the status of these segments in Fn.

Ionic Strength as a Modulator of Epitope Expression. Hydrodynamic studies have shown that Fn undergoes reversible conformational changes as a function of ionic strength (Alexander et al., 1979; Williams et al., 1982; Rocco et al., 1983; Sjoberg et al., 1989; Benecky et al., 1991). Accordingly, we sought to determine whether ionic strength conditions would modulate the surface availability of the epitopes within the 9th and 10th type III modules of Fn recognized by mAbIII-9 and mAbIII-10. In the initial analysis, a competitive ELISA was used in which the capacity of soluble Fn to inhibit the binding of the mAb to immobilized Fn was measured. Changes in ionic strength had no effect on the binding of each mAb to immobilized Fn (Figure 3); thus, changes in the inhibitory potency of soluble Fn reflect differences in exposure of its epitopes. In this system at μ 0.15, soluble Fn, at 10 μg/mL, produced 40−60% inhibition of mAbIII-9 and III-10 binding to the immobilized Fn (Figure 3). When the ionic strength was increased to 1.0 by addition of NaCl, greater inhibition was observed, indicating increased exposure of the target epitopes of both mAb in soluble Fn. In contrast, at μ 0.01, the inhibitory capacity of soluble Fn was eliminated, indicating that the mAb could no longer react with their epitopes. mAbIII-4 showed a very different pattern of reactivity: maximal and similar epitope expression was observed at the ionic strength extremes with modest modulation at intermediate ionic strengths. The binding of mAb 8A11 was the same at μ 0.01 and 1.0 (not shown).

The effects of ionic strength on epitope availability were evaluated further in a fluid phase radioimmunoassay. In this assay, the binding of varying amounts of soluble ^{125}I-Fn by a constant amount of mAb was assessed. Scatchard plots were constructed from binding data obtained at μ 0.15 and 1.0. As shown in Figure 4 and in Table 1, the binding constant (K_a) for the interaction of Fn with mAbIII-10 differed at the two ionic strengths. At μ 0.15, the K_a was $(5.0 \pm 0.9) \times 10^8$ M^{-1} compared to $(21 \pm 2) \times 10^8$ M^{-1} at μ 1.0. Notable is the stoichiometry of mAbIII-10 binding. Rather than the predicted value of 2 molecules of mAb bound per molecule of Fn at saturation (one epitope per each Fn subunit), the stoichiometry approached a 1:1 ratio. This stoichiometry was similar at both low and high ionic strength: 1.23 at μ 0.15 and 1.32 at μ 1.0. (The stoichiometry mAb/Fn was calculated from the reciprocal of the Fn/mAb ratio obtained from extrapolation of the Scatchard plots.) The behavior of the mAbIII-10 epitope was unique. As shown in Table 1, with the three other mAb tested, their stoichiometry approached values of two epitopes per Fn molecules.

Fn Ligands as Modulators of Epitope Expression. The functions of Fn are, in part, dependent upon its capacity to interact with a variety of ligands. Among these, glycosaminoglycans bind to Fn and influence cell adhesion. Accordingly, we have analyzed the effects of selected glycosaminoglycans on expression of the epitopes for mAbIII-9 and mAbIII-10. As evaluated in the ELISA format, heparin and heparan sulfate increased the capacity of soluble Fn to inhibit

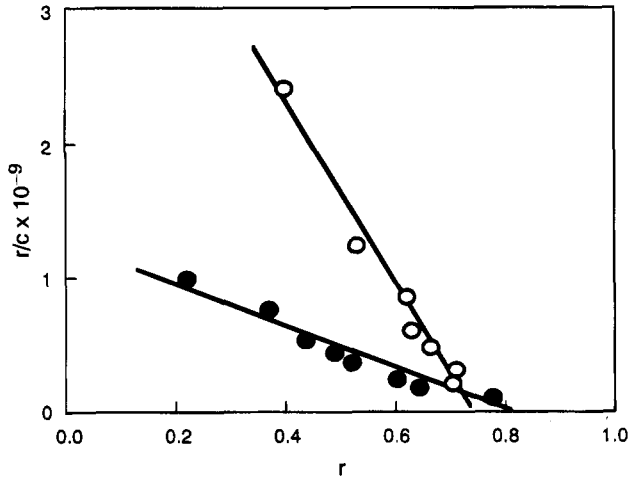

FIGURE 4: Scatchard plots of binding data for the interaction of mAbIII-10 with soluble Fn at ionic strengths of 0.15 (●) and 1.0 (○). The primary binding data were derived in a radioimmunoassay in which a fixed concentration of antibody (1 nM) was incubated with ^{125}I-Fn at concentrations of 0.5−20 nM (deviation from linearity was observed at Fn concentrations >50 nM, presumably due to self-association of Fn). The experiment shown is representative of five separate determinations with two different preparations of Fn. r = [bound Fn]/[total antibody]; c = [free Fn].

Table 1: Binding Parameters for Interaction of Fn with Monoclonal Antibodies in Solution[a]

	$K_a \times 10^8$ (M^{-1})		stoichiometry (mAb/Fn)	
mAb	μ 0.15	μ 1.0	μ 0.15	μ 1.0
mAbIII-10	5.0 ± 0.9	21.0 ± 2.0	1.23 ± 0.05	1.32 + 0.01
mABIII-9	11.0 ± 2.2	19.7 ± 4.2	2.2 ± 0.28	2.20 ± 0.05
mAbIII-4	19.2 ± 2.8	24.1 ± 2.9	1.84 ± 0.08	2.0 ± 0.15
mAb8A11	11.1 ± 1.0	ND	2.0 ± 0.1	ND

[a] Values are the means ± SD of 2−5 determinations. ND = not determined.

mAbIII-10 binding to the immobilized Fn target, indicative of greater accessibility to the mAbIII-10 epitope (Figure 5A). Both substances affected expression of the mAbIII-9 epitope only modestly. For example, at 0.01 mg/mL heparin the soluble Fn (10 μg/mL) inhibited mAbIII-10 binding by 55%, whereas it required 0.25 mg/mL heparin to attain the same effect on mAbIII-9 binding. To ensure that the effects of heparin were confined to modulation of epitope expression in the soluble rather than the immobilized Fn, the experiments were repeated with the 110 kDa chymotryptic fragment coated onto the microtiter plates. As this fragment lacks both major heparin binding sites, effects of heparin are restricted to the soluble Fn competitor, and heparin did not affect the reaction of mAbIII-10 and mAbIII-9 with the immobilized 110K fragment. In this assay format, the same results were obtained; namely, heparin modulated expression of the epitope within the 10th and to a lower extent within the 9th type III repeat. By RIA, the effect of heparin on mAbIII-10 binding to Fn was assigned to an increase in affinity. In the presence of 16 μg/mL heparin, the K_a of mAbIII-10 for Fn increased by 2.5-fold (from 5×10^8 M^{-1} in the absence of heparin to 1.26×10^9 M^{-1} in the presence of heparin) without altering the stoichiometry of binding. Two other glycosaminoglycans, chondroitin sulfates A and B, which react minimally with Fn (Yamada et al., 1980), affected expression of mAbIII-10 epitope only slightly and were without effect on the mAbIII-9 epitope (Figure 5A). In addition, hyaluronic acid, either in monomeric or in polymeric form, did not affect the exposure of the two modules.

Ca^{2+} binds to Fn (Amphlett & Hrinda, 1983) and modulates the interaction of heparin with specific domains of Fn (Hayashi & Yamada, 1982). In competitive ELISA, at concentrations up to 10 mM, Ca^{2+} had little effect on expression of the mAbIII-10 epitope (Figure 5B). However, Ca^{2+} did eliminate the effect of heparin on exposure of the 10th segment. Soluble Fn, at 10 μg/mL in the presence of 50 μg/mL heparin, decreased the binding of mAbIII-10 to immobilized Fn about 90%, whereas it produced only 10% inhibition in the absence of heparin (Figure 5B). Increasing concentrations of Ca^{2+} progressively neutralized the effect of heparin, with a half-maximal effect at 4 mM.

Gangliosides bind to Fn and influence the capacity of cells to assemble a Fn matrix (Thompson et al., 1986; Spiegel et al., 1986). Several members of the ganglioside family were tested for their effects on Fn conformation. As shown in Figure 5C, sialylated gangliosides G_{M1}, G_{D1A}, and G_{T1b} affected the exposure of 10th type III segment markedly as indicated by their influence on mAbIII-10 binding. These gangliosides had only minimal effects on the expression of 9th segment. For example, gangliosides G_{D1a} and G_{M1} at 0.25 mg/mL increased the inhibition by 10 μg/mL soluble Fn of mAbIII-10 binding to immobilized Fn by 70%, whereas the same concentration of these gangliosides enhanced the inhibition of mAbIII-9 binding by Fn by only 20%. In contrast, desialylated ganglioside G_{M3} had minimal effect on the exposure of the 10th segment. The effect of gangliosides can be placed in order $G_{T1b} > G_{D1a}$, $G_{M1} > G_{M3}$.

Interaction of Fn with collagens is of major importance in matrix assembly. Accordingly, the effect of gelatin on epitope expression in the 9th and 10th modules was assessed. As shown in Figure 5D, gelatin augmented the capacity of soluble Fn to inhibit mAbIII-10 binding to immobilized Fn. This effect was dependent upon the concentration of gelatin. The exposure of epitope in the 9th module was affected by gelatin to a lesser extent. Gelatin at 0.25 mg/mL increased the inhibitory effect of soluble Fn on mAbIII-10 and mAbIII-9 binding to immobilized Fn by 50% and 20%, respectively.

Multimerization as a Modulator of Epitope Expression. When purified Fn was applied to a Sephacryl S-300 column, two fractions were discerned: a minor peak (I) of higher Stokes' radius and a major peak (II) of lower Stokes' radius (Figure 6A). Individual fractions were analyzed for mAbIII-10 epitope expression by RIA. Data obtained with three representative fractions are shown in the inset to Figure 6A. A fraction from the ascending portion of peak II (fraction 21) had a higher affinity for mAbIII-10 than one (fraction 28) from the descending portion. A fraction from peak I (fraction 18) had a still higher affinity for mAbIII-10. The difference in affinity of fractions 18 and 28 was more than 5-fold. Control experiments showed that gel filtration itself did not induce formation of Fn molecules with increased epitope expression. Upon rechromatography of Fn from the descending part of peak II (fraction 25), its elution volume (V_e) and apparent affinity for mAbIII-10 were not changed.

A molecular basis for the differential expression of the mAbIII-10 epitope by these fractions was sought. SDS−PAGE of these fractions revealed no cross-linked, high molecular weight Fn oligomers or degradation products.

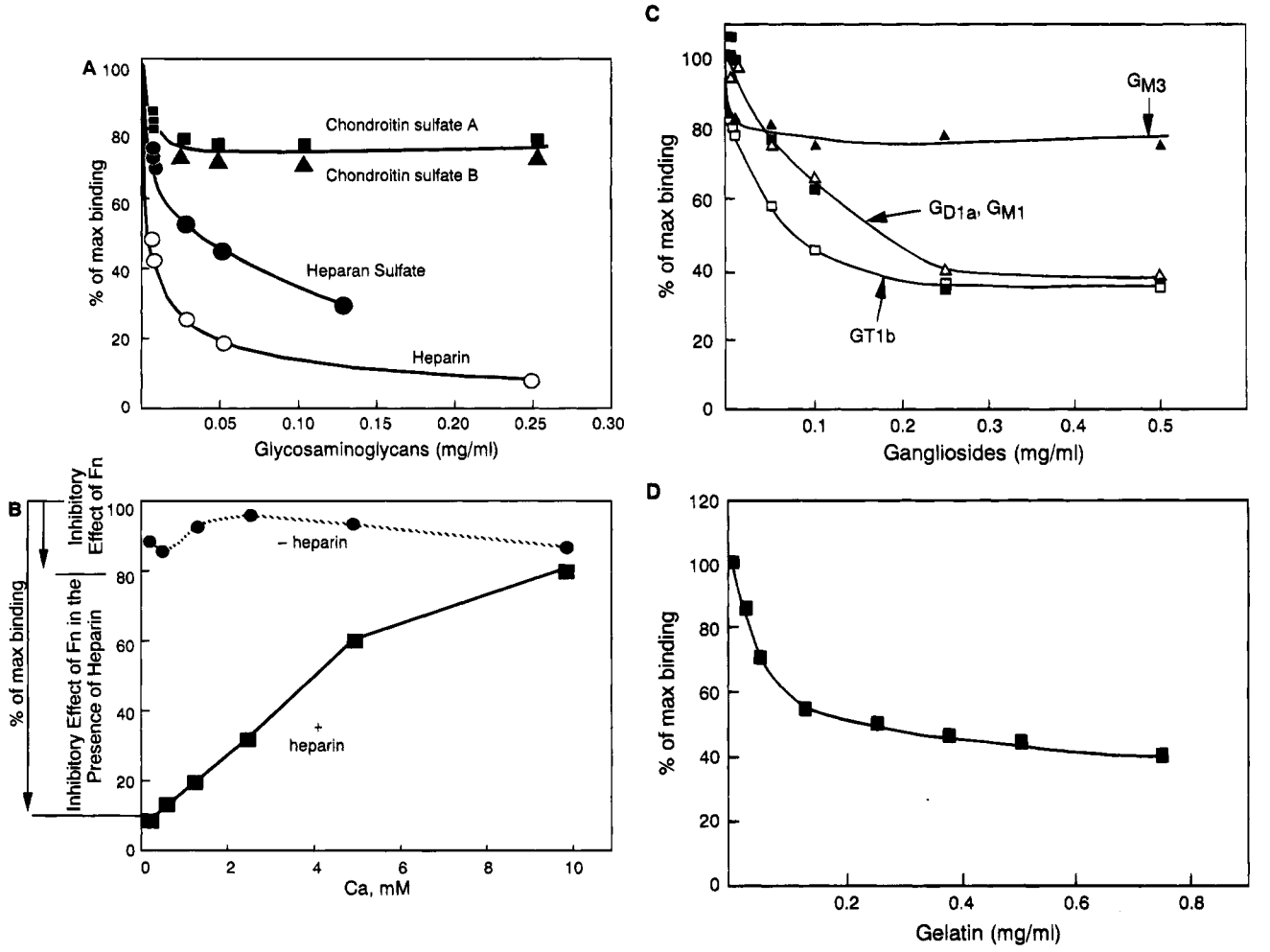

FIGURE 5: Effect of Fn ligands on the expression of 10th type III modules. Panels A, C, and D: Fn (10 μg/mL) was mixed with various ligands at the indicated concentrations in PBS for 30 min at 22 °C. mAbIII-10 (1 μg/mL final concentration) was then added, and the mixture was transferred into Fn-coated microtiter wells in a competitive ELISA format. The binding of antibody in the presence of Fn (10 μg/mL) was assigned a value of 100% binding. Ligands themselves did not affect the exposure of epitope in the immobilized Fn. At the highest concentrations of heparin, heparan sulfate, and chondroitin sulfates A and B used, binding of mAbIII-10 was 102%, 99%, 93%, and 100% of the buffer control. Binding of mAb in the presence of all gangliosides and gelatin was 100% and 96%, respectively. The experiments shown are representative of 2−3 independent determinations for each ligand. Panel B: Soluble Fn (10 μg/mL) was incubated with heparin (50 μg/mL) alone or in the presence of increasing concentrations of Ca^{2+} for 30 min at 22 °C. The experiment then proceeded as above. In this experiment, the binding of the mAbIII-10 to immobilized Fn in the absence of soluble Fn competitor was assigned a value of 100%. Ca^{2+} did not affect the inhibitory capacity of Fn (●) in the absence if heparin.

Differences in the extent of noncovalent multimerization of Fn within the fractions were assessed by adding the water-soluble bifunctional reagent, bis(sulfosuccinimidyl) suberate. In dilute protein solutions, this reagent should chiefly cross-link noncovalently associated protein molecules to form covalent complexes, without covalently cross-linking non-complexed molecules. As shown in Figure 6B, all the Fn in fractions from peak I (fractions 17, 19, and 20) were converted to high molecular weight aggregates. The tendency of Fn to form cross-linked aggregates remained high in fractions from the ascending part of peak II and declined in the descending part of peak II. Thus, Fn multimerization is correlated with increased affinity for mAbIII-10.

Surface as a Modulator of Epitope Expression. As assessed by electron microscopy, interaction of Fn with substratum induces profound changes in shape (Erickson et al., 1981; Erickson, 1985; Price et al., 1982). Accordingly, we tested the effect of Fn deposition on the expression of the epitopes in 9th and 10th type III modules. Fn was coated on Immulon 2 plates, and the affinity and stoichiometry of mAbIII-10 and mAbIII-9 binding to the immobilized Fn were determined using labeled mAb (Table 2). The affinity constants (K_a) for mAbIII-10 and mAbIII-9 for the immobilized Fn were similar to the values measured in solution. However, deposition of Fn onto the surface did change the stoichiometry of mAb binding. For MabIII-10, 2.2 ± 0.1 molecules bound per deposited Fn molecule compared to the 1:1 stoichiometry in solution (Table 2). For mAbIII-9, 0.6 ± 0.06 molecules bound per Fn molecule compared to a stoichiometry of 2.2 for Fn in solution. Thus, the exposure of the 9th and 10th modules was differentially influenced by surface deposition of Fn. The stoichiometry of mAbIII-4 binding was 1.8 ± 0.1 (Table 2), similar to the value of 1.84 observed in solution, but the affinity of this mAb for immobilized Fn was less.

Electron Microscopy of mAbIII-10 Bound to Fn. To further understand the basis for the extensive modulation of the mAbIII-10 epitope, we sought to visualize complexes of mAbIII-10 and soluble Fn by electron microscopy. In the absence of mAb, Fn molecules appeared in a variety of shapes (Figure 7a−c), consistent with previous reports (Erickson & Carrel, 1983; Engel et al., 1981). To character-

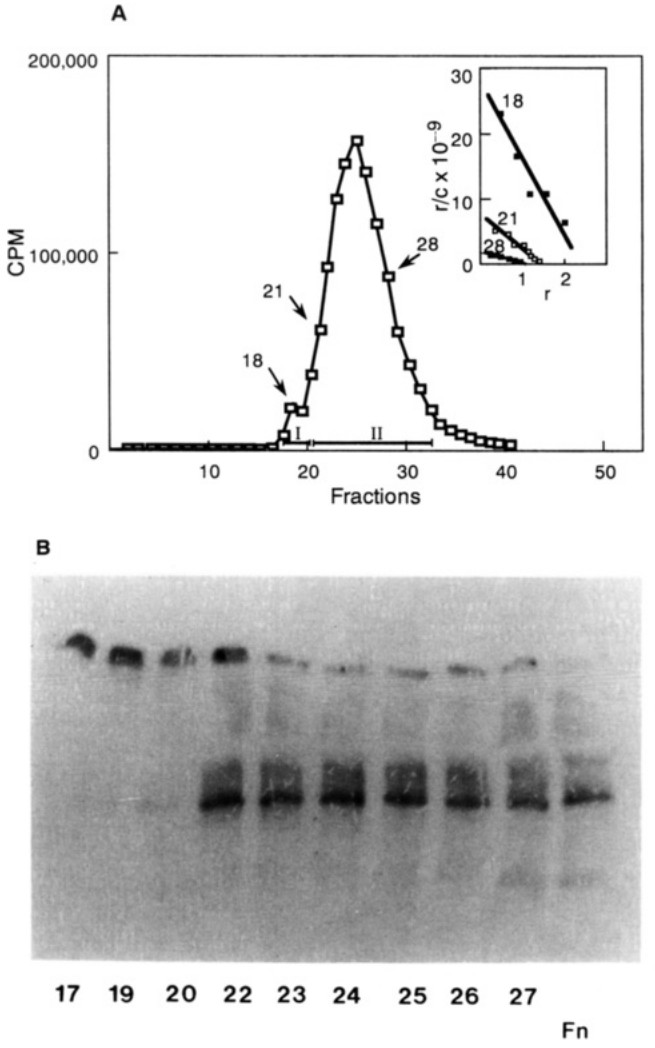

FIGURE 6: Heterogeneity of mAbIII-10 epitope expression in purified plasma Fn. Panel A: Gel filtration of purified ^{125}I-Fn on Sephacryl S-300 (1 × 90 cm), equilibrated with PBS. Fractions (0.6 mL) were collected and counted in a γ-counter, and the absorbance at 280 nm was measured. Then fractions were supplemented with 1% BSA. Positions of peaks I and II are indicated by the horizontal bars. Inset: Scatchard analysis of binding of mAbIII-10 with three fractions of Fn from different parts of the elution profile (shown by arrows). The axes are as in Figure 4. Panel B: The cross-linking reagent, bis(sulfosuccinimidyl) suberate, was added to the gel filtration fractions at a 100-fold molar excess of protein. After incubation for 1 h at 22 °C, the mixtures were subjected to SDS-PAGE on 5% acrylamide gels (3.5% stacking gel) without reduction, and reaction products were visualized by autoradiography. The cross-linked species entered the 3.5% stacking gel and migrated more slowly than an IgM standard (not shown).

Table 2: Binding Parameters for the Interaction of Antibodies with Surface-Bound Fn

mAb	$K_a \times 10^8$ (M^{-1})	stoichiometry (mAb/Fn)
mAbIII-10	3.7 ± 0.28	2.2 ± 0.1
mAbIII-9	12.7 ± 0.7	0.6 ± 0.6
mAbIII-4	5.2 ± 0.07	1.8 ± 0.1

ize quantitatively the different Fn conformations, we analyzed more than 300 individual molecules from randomly chosen fields and classified them into the three following groups: compact, tangled structures (Figure 7a); V-shaped structures (Figure 7b); and unfolded strands (Figure 7c). Of the molecules observed, 13% were in the compact form, 22% had a V-shape, and 65% appeared as partially or completely unfolded strands.

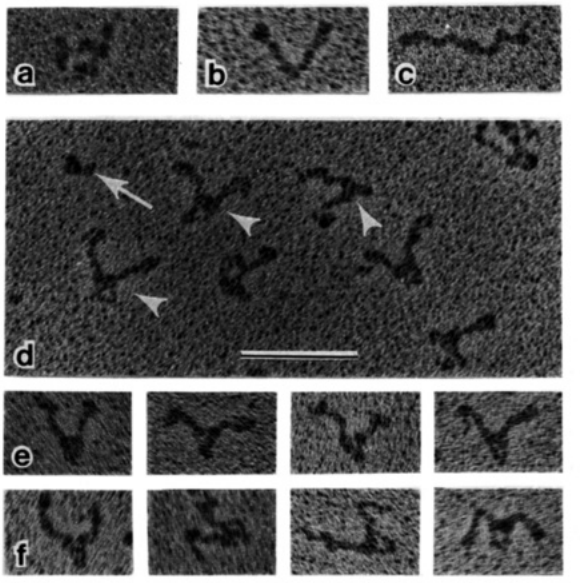

FIGURE 7: Electron micrographs of fibronectin and fibronectin–mAbIII-10 complexes. All specimens are rotary shadowed with tungsten. Panels a–c are selected examples of individual fibronectin molecules in the compact, tangled (a), V-shaped (b), and unfolded (c) conformations. Panels d–f are a field and selected examples of fibronectin–antibody complexes. The arrowheads identify the most commonly observed species, in which mAbIII-10 bound between two arms of the V-shaped fibronectin. The individual mAb molecules (arrow) usually appear as structures with three flexible lobes. Bar represents 100 nm.

mAbIII-10 was incubated with Fn for 4 h at 22 °C, and after dilution, the mixture was sprayed and shadowed with tungsten. Many complexes formed between Fn and mAbIII-10. The conformation of Fn in these complexes was very different than in the absence of mAb: 79% of all Fn molecules had a V-shaped structure, compared to 22% in the absence of mAb; 8% were unfolded, and 13% were in the compact form. Almost all V-shaped Fn molecules had one and only one mAb bound, and each Fab lobe appeared to contact on an arm of the Fn dimer. In other words, the mAb appeared to lie between the two arms of the "V" of Fn (Figure 7d–f). Thus, the epitope for mAbIII-10 appears to reside on the inner surface of the Fn arms close enough to be spanned by the Fab regions of the same antibody. Images in which the mAb bound to the outer surface of the Fn arms were never found. In rare images in which two mAb bound per Fn, the Fn molecule was in an extended conformation. In mixtures of Fn with mAbIII-9 and mAbIII-4, the conformational distributions of Fn molecules were similar to those seen in control samples, and complexes of Fn with the mAb were rarely observed. In most cases, the molecules of Fn and mAb were lying near to each other at short distances, suggesting that they had dissociated during the preparation of samples for electron microscopy. On rare occasions, large aggregates of Fn and mAb were observed, indicating that more than one antibody bound to each Fn. Such aggregates could arise from the cross-linking of Fn molecules by the bivalent antibodies.

DISCUSSION

In this study, we have shown that the availability of epitopes within the 9th and 10th type III homology segments

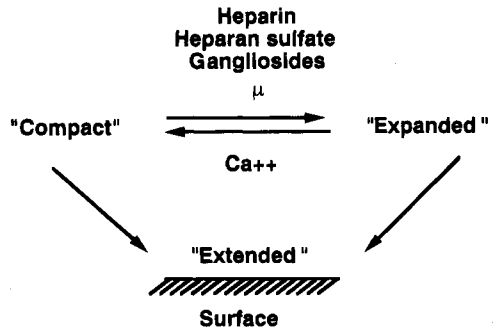

FIGURE 8: Conformational states of fibronectin regulate availability of epitopes within the 10th type III repeats. Fibronectin exists in three distinguishable conformations. In the "compact" conformation the mAbIII-10 epitopes are minimally available. Binding of heparin, heparan sulfate, or ganglioside induces a reversible transition of fibronectin from a "compact" conformation to an "expanded" conformation. This change increases the availability of III-10 epitope and, as a consequence, the affinity of mAb. The V-shaped Fn is one of the expanded conformers. Deposition of fibronectin on a surface unfolds the molecule to an "extended" conformation, exposing both copies of the epitope.

of plasma Fn can be greatly modulated. These two repeats contain sequences that have been directly implicated in the cell adhesive properties of Fn; and the mAb developed to each of these segments did, indeed, inhibit platelet adhesion to Fn. Thus, the conformational transitions of Fn, evoked by microenvironmental conditions and by its interaction with physiological ligands, have the potential to be a significant regulator of the adhesive properties of Fn.

The 10th type III repeat, which contains the RGD sequence recognized by multiple integrin adhesion receptors, was particularly susceptible to conformational modulation. A variety of components in extracellular matrices and on cell surfaces, including heparin, heparan sulfate, gangliosides G_{M1}, G_{DA1}, and G_{T1b}, and gelatin, increased the accessibility of epitope recognized by mAbIII-10. Specificity was, however, observed since chondroitin sulfates A and B and hyaluronic acid did not affect the expression of the module. The increased accessibility of the 10th module to its mAb appears to arise as a consequence of partial unfolding of Fn, resulting in a significant change in molecular shape. This prediction is supported by the fact that the effects of these various ligands on epitope expression, i.e., to increase epitope exposure, were simular to that induced by increasing ionic strength. It is well-established that 1 M NaCl modulates the shape of Fn. Hydrodynamic studies showed that Stokes' radius of Fn increases from 8.6−10.5 nm at μ 0.15 to 16−17.5 nm at μ 1.0 (Williams et al., 1982; Rocco et al., 1983). Additionally, the effects of heparin on the conformation of Fn have been previously described as a transition of the molecule to a more "relaxed" state (Ankel et al., 1986). Several of the effector ligands bind at sites within Fn that are spatially quite distant from one another and from the 10th type III repeat in terms of linear amino acid sequence. This transmission of conformational changes over extended distances is also compatible with substantial changes in the protein shape. Nevertheless, the unfolding of Fn must be partial since full expression, the availability of two copies of the epitope such as attained with Fn on a surface, was not observed. Taken together, these observations are consistent with the interpretation of Lai et al. (1993) and support the model depicted in Figure 8. Accordingly, interaction of Fn with ligands or exposure to high ionic strength can cause the molecule to undergo a molecular "expansion". Such an expansion results in greater availability of the 10th type III repeat at the hydrated surface of Fn. This molecular expansion also affects the recognized epitope in 9th type III repeat although the influence on this region is not as extensive. This expansion is distinct from the "extension" of the molecule when Fn interacts with an appropriate surface (see below).

Two of the conditions that influenced this partial unfolding of the molecule merit specific comment. First, soluble heparin and heparan sulfate induced unfolding of Fn to enhance availability of the 10th type III module. These data suggest that cell surface and matrix heparan sulfate may also modulate Fn conformation. It was proposed that heparan sulfate containing proteoglycans may alter the conformation of certain growth factors so that they acquire high affinity for their receptor (Yayon et al., 1991; Ruoslahti & Yamaguchi, 1991). Furthermore, recently it has been shown that exposure of RGDS sequence in thrombin by plasmin was significantly enhanced in the presence of soluble heparin and heparan sulfate, but not other glycosaminoglycans (Bar-Shavit et al., 1993).

Ca^{2+} eliminated the unfolding induced by heparin, probably by stabilizing the structure of Fn. In accordance with this interpretation, the heparin-induced precipitation of plasma Fn at 4 °C is completely prevented by 10 mM $CaCl_2$ (Richter et al., 1985). Stabilization of structure by Ca^{2+} is a common characteristic among adhesive proteins. For example, Ca^{2+} stabilizes the structures of fibrinogen and thrombospondin to proteolysis (Belitser et al., 1975; Lawler et al., 1982).

Second, the purified Fn was heterogeneous with respect to noncovalent self-association, as evaluated by cross-linking analyses, and this heterogeneity correlated with differences in expression of the 10th module, i.e., differences in affinity for mAbIII-10. Self-assembly may directly induce exposure of the epitope. Alternatively, the increased availability of the epitope may be the consequence of the purification procedure which exposes Fn to gelatin and 1 M arginine. Self-association may then prevent the full return of the epitope to its state of least exposure. In either case, these data suggest that isolated Fn may be functionally heterogeneous and that self-association of Fn, such as would occur in a matrix, may influence its adhesive properties.

The limited exposure of the epitope within the 10th type III repeat in soluble Fn is supported by the observation that only one mAbIII-10 bound per Fn molecule in solution as determined by Scatchard analyses. In contrast, a 2:1 stoichiometry was found for three other antibodies (Table 1). The binding of one mAbIII-10 molecule per one Fn molecule was confirmed by visualization of the complex by electron microscopy. That each subunit of the dimeric Fn molecule contains one mAbIII-10 epitope is indicated by the binding of two mAbIII-10 per Fn deposited on the microtiter well surface. Moreover, in Western blots, mAbIII-10 reacted with both the 113 and 146-kDa tryptic fragments, which originate from large and small subunits of Fn, respectively (data not shown). Thus, to account for the binding of a single antibody to soluble Fn, two possible explanations may be considered: (1) The Fn molecule is asymmetric, and only one 10th module is exposed on the surface of Fn. Taking into consideration that plasma Fn exists predominantly as a heterodimer consisting of two subunits differing by the

presence of alternatively spliced V-region, an asymmetric fold of the Fn subunits is conceivable. (2) The two 10th type III modules within the Fn molecule may be juxtaposed such that binding of a single antibody molecule precludes access of a second antibody. Such an exclusion could arise from steric considerations or from engagement of both epitopes by the bivalent antibody. The distance between SH_1 groups in two 7th type III homology segments (regions adjacent to 10th modules) was estimated to be 3.5−4.4 nm (Wolff & Lai, 1990), and the distance between the antigen binding sites within a single IgG molecule varies from about 5 to 14 nm because of the flexible hinge region (Valentine & Green, 1967; Silverton et al., 1977). Thus, such bridging of epitopes by a single antibody could possibly occur. While the use of Fab fragments might address these steric possibilities, the access to this region of biologically relevant macromolecules may be more pertinent. The images of Fn associated with GPIIb-IIIa usually showed binding of only one Fn molecule to the receptor (Weisel et al., 1992). Under similar conditions, fibrinogen, also a dimeric molecule, was sometimes engaged by two receptors. Furthermore, in the electron micrographs obtained by Gailit and Ruoslahti (1988), only complexes consisting of one molecule of purified receptor $\alpha_5\beta_1$ with one molecule of Fn were seen.

In contrast to the soluble form, Fn immobilized onto a plastic surface bound two molecules of mAbIII-10 (Table 2). Thus, exposure of the second epitope in surface-bound Fn could be associated with a more global and extensive unfolding of the molecule (see Figure 8). Two lines of evidence support this interpretation. In electron micrographs, Fn is heterogeneous and can often be visualized as an extended rod-like structure (Erickson et al., 1981; Price et al., 1982). One such structure is shown in Figure 7c. It has been suggested that the nature of the substratum and the method of sample preparation for electron microscopy may be the factors that contribute to such unfolding of Fn (Erickson, 1985). Furthermore, it has been shown by fluorescence energy transfer that the two amino-terminal and SH_1-containing regions are close in soluble Fn but become more distant upon binding of the molecule to the surface of Cytodex beads (Wolff & Lai, 1989, 1990). Taken together, such data distinguish surface-induced elongation of Fn from the expansion of the molecule induced by the various effector ligands. The differential effects of ligands and surface deposition on the mAbIII-9 epitope also support this conclusion. Ligands increased accessibility of this epitope to its mAb, whereas surface deposition diminished access. Differential epitope expression upon deposition on different surfaces has been observed with other antibodies to Fn (Grinnell & Feld, 1982; Underwood et al., 1993).

While the nature of the conformational changes induced by surface deposition and ligand interactions may be different, the functional consequences of molecular expansion and elongation on the adhesive properties of Fn appear to be similar. Both types of changes result in increased accessibility of the 10th type III repeat and, therefore, potentially increased adhesive functions. Exposure of the second 10th module by unfolding of surface-bound Fn may be at least partially responsible for increased adhesiveness of immobilized Fn, the "surface activation of plasma Fn" (Pearlstein, 1978; Grinnell et al., 1982; Colvin & Kradin, 1983; Schwartz & Juliano, 1984). The molecular expansion of Fn resulting in increased accessibility of 10th type III module(s) of Fn may explain why heparin, heparan sulfate, and collagen transform soluble Fn to a more effective inhibitor of cell attachment to surface-bound Fn (Johansson & Höök, 1984). Thus, in addition to possessing the appropriate primary sequence within the context of an appropriate secondary structure (e.g., RGDS in a β turn [Main et al., 1992]), presentation of the sequence in an optimal tertiary and quaternary structure may control the adhesive properties of Fn.

The present study supports and extends the concept that adhesive proteins expose additional binding sites upon interaction with an appropriate surface. The adhesive proteins may, as a group, be organized into structures which permit ready and global unfolding. Previously, we have shown that deposition of fibrinogen onto plastic or onto its receptor on platelets evoked unmasking of an RGDF sequence in its Aα chain (Ugarova et al., 1993). Recognition of von Willebrand factor by GPIb on platelets also appears to be conformationally determined (Moake et al., 1986; Ikeda et al., 1991). It is now appreciated that the interaction of many integrins with adhesive proteins is regulated by the conformational state of the receptors (Plow & Ginsberg, 1981; Kovach et al., 1992; Masumoto & Hemler, 1993; Faull et al., 1993). The conformational flexibility and states of the adhesive ligands may also play an important regulatory role in controlling cell adhesion.

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REFERENCES

Alexander, S. S., Jr., Colonna, G., & Edelhoch, H. (1979) *J. Biol. Chem. 254*, 1501−1505.
Amphlett, G., & Hrinda, M. E. (1983) *Biochem. Biophys. Res. Commun. 111*, 1045−1053.
Ankel, E. G., Homandberg, G., Tooney, N. M., & Lai, C.-S. (1986) *Arch. Biochem. Biophys. 244*, 50−56.
Bar-Shavit, R., Eskohjido, Y., Fenton, J. W., III, Esko, J. D., & Vlodavsky, I. (1993) *J. Cell. Biol. 123*, 1279−1287.
Belitser, V. A., Varetskaja, T. V., Tolstykh, V. M., Tsarjuk, L. A., & Pozdnjakova, T. M. (1975) *Thromb. Res. 7*, 797−806.
Benecky, M. J., Wine, R. W., Kolvenbach, C. G, & Mosesson, M. W. (1991) *Biochemistry 30*, 4298−4306.
Bevilacqua, M. P., Amrani, D., Mosesson, M. W., & Bianco, C. (1981) *J. Exp. Med. 153*, 42−60.
Bowditch, R. D., Halloran, C. E., Aota, S., Obara, M., Plow, E. F., Yamada, K. M., & Ginsberg, M. (1991) *J. Biol. Chem. 266*, 23323−23328.
Bowditch, R. D., Hariharan, M., Tominna, E. F., Smith, J. W., Yamada, K. M., Getzoff, E. D., & Ginsberg, M. H. (1994) *J. Biol. Chem. 269*, 10856−10863.
Colvin, R. B., & Kradin, R. L. (1983) *Surv. Synth. Pathol. Res. 2*, 10−20.
Czop, J. K., Kadish, J., & Austen, K. F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3649−3653.
DePetro, G., Barlati, S., Vartio, T., & Vaheri, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4965−4969.
Engel, J., Odermatt, E., Engel, A., Madri, J. A., Furthmayr, H., Rohde, H., & Timple, R. (1981) *J. Mol. Biol. 150*, 97−120.
Erickson, H. P. (1985) in *Plasma Fibronectin Structure and Function* (McDonagh, J., Ed.) pp 31−51, Marcel Dekker, New York.
Erickson, H. P., & Carrel, N. (1983) *J. Biol. Chem. 258*, 14539−14544.
Erickson, H. P., Carell, N., & McDonagh, J. (1981) *J. Cell Biol. 91*, 673−678.

Faull, R. J., Kovach, N. L., Harlan, J. M., & Ginsberg, M. H. (1993) *J. Cell Biol. 121*, 155−162.
Gailit, J., & Ruoslahti, E. (1988) *J. Biol. Chem. 263*, 12927−12932.
Grinnell, F. (1980) *J. Cell Biol. 86*, 104−112.
Grinnell, F., & Feld, M. (1982) *J. Biol. Chem. 257*, 4888−4893.
Grinnell, F., Lang, B. R., & Phau, T. V. (1982) *Exp. Cell Res. 142*, 499−504.
Hayashi, M., & Yamada, K. (1982) *J. Biol. Chem. 257*, 5263−5267.
Homandberg, G. A., & Erickson, J. W. (1986) *Biochemistry 25*, 6917−6925.
Homandberg, G., Williams, J. E., Grant, D., Schumacher, B., & Einstein, R. (1985) *Am. J. Pathol. 120*, 327−332.
Hörmann, H., & Richter, H. (1986) *Biopolymers 25*, 947−958.
Hynes, R. O. (1990) *Fibronectins*, Springer-Verlag, New York.
Ikeda, Y., Handa, M., Kawano, K., Kamata, T., Murata, T., Araki, Y., Anbo, H., Kawai, Y., Watanabe, K., Itagaki, I., Sakai, K., & Ruggeri, Z. M. (1991) *J. Clin. Invest. 87*, 1234−1240.
Johansson, S., & Höök, M. (1984) *J. Cell Biol. 98*, 810−817.
Kovach, N. L., Carlos, T. M., Yee, E., & Harlan, J. (1992) *J. Cell Biol. 116*, 499−509.
Laemmli, U. K. (1970) *Nature (London) 22*, 680−685.
Lai, C.-S., Wolff, C. E., Novello, D., Griffone, L., Cuniberti, C., Molina, F., & Rocco, M. (1993) *J. Mol. Biol. 230*, 625−640.
Lawler, J., Chao, F. C., & Cohen, C. M. (1982) *J. Biol. Chem. 257*, 12257−12265.
Litvinovich, S. V., Strickland, D. K., Medved, L. V., & Ingham, K. L. (1991) *J. Mol. Biol. 217*, 563−575.
Main, A. L., Harvey, T. S., Baron, M., Boyd, J., & Campbell, I. D. (1992) *Cell 71*, 671−678.
Masumoto, A., & Hemler, M. E. (1993) *J. Biol. Chem. 268*, 228−234.
Matsuka, Y. V., Medved, L. V., Brew, S. A., & Ingham, K. L. (1994) *J. Biol. Chem. 269*, 9539−9546.
Moake, J. L., Tutner, N. A., Stathopoulos, N. A., Nolasco, L. H., & Hellums, J. D. (1986) *J. Clin. Invest. 78*, 1456−1461.
Mosher, D. F., Ed. (1989) *Fibronectin*, Academic Press, San Diego, CA.
Muir, D., & Manthorpe, M. (1992) *J. Cell Biol. 116*, 177−185.
Norris, D. A., Clark, R. A. F., Swigart, L. M., Huff, J. C., & Weston, W. L. (1982) *Immunology 129*, 1612−1618.
Pearlstein, E. (1978) *Int. J. Cancer 22*, 32−35.
Plow, E. & Ginsberg, M. (1981) *J. Biol. Chem. 256*, 9477−9482.
Price, T., Rudee, M., Pierschbacher, M., & Ruoslahti, E. (1982) *Eur. J. Biochem. 129*, 359−363.
Rennard, S. I., Wind, M. L., Hewitt, A. T., & Kleinman, H. K. (1981) *Arch. Biochem. Biophys. 206*, 205−212.
Richter, H., Wendt, C., & Hörmann, H. (1985) *Biol. Chem. Hoppe Seyler 366*, 509−514.
Robinson, R. M., & Hermans, J. (1984) *Biochem. Biophys. Res. Commun. 125*, 718−725.
Rocco, M., Carson, M., Hantgan, R., McDonagh, J., & Hermans, J. (1983) *J. Biol. Chem. 258*, 14545−14549.
Ruoslahti, E. (1988) *Annu. Rev. Biochem. 57*, 375−413.
Ruoslahti, E. (1991) *J. Clin. Invest. 87*, 1−5.
Ruoslahti, E., & Yamaguchi, Y. (1991) *Cell 64*, 867−869.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660−672.
Schwartz, M. A., & Juliano, R. L. (1984) *Exp. Cell Res. 152*, 302−312.
Sekiguchi, K., Hakomori, S., Funahasi, M., Matsumoto, I., & Seno, N. (1983) *J. Cell Biol. 258*, 14359−14365.
Shadle, P. J., Ginsberg, M. H., Plow, E. F., & Barondes, S. H. (1984) *J. Cell Biol. 99*, 2056−2060.
Shattil, S. J., Cunningham, M., & Hoxie, J. A. (1987) *Blood 70*, 307−315.
Silvertone, E. W., Naria, M. A., & Davies, D. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5140−5144.
Sjöberg, B., Eriksson, M., Österlund, E., Pap, S., & Österlund, K. (1989) *Eur. Biophys. 17*, 5−11.
Spiegel, S., Yamada, K. M., Hom, B. E., Moss, J., & Fishman, P. H. (1986) *J. Cell. Biol. 102*, 1898−1906.
Thompson, L. K., Horowitz, P. M., Bentley, K. L., Thomas, D. D., Alderete, J. F., & Klebe, R. J. (1986) *J. Biol. Chem. 261*, 5209−5214.
Tooney, N. M., Mosesson, M. W., Amrani, D. L., Hainfeld, J. F., & Wall, J. S. (1983) *J. Cell Biol. 97*, 1686−1692.
Ugarova, T. P., Budzynski, A. Z., Shattil, S. J., Ruggeri, Z. M., Ginsberg, M., & Plow, E. F. (1993) *J. Biol. Chem. 268*, 21080−21087.
Underwood, P. A., Steele, J. G., & Dalton, B. A. (1993) *J. Cell Sci. 104*, 793−803.
Valentine, R. C., & Green, N. M. (1967) *J. Mol. Biol. 27*, 615−617.
Veklich, Y. I., Gorkun, D. V., Medved, L. V., Nieuwenhuizen, W., & Weisel, J. W. (1993) *J. Biol. Chem. 268*, 13577−13585.
Vuento, M., & Vaheri, A. (1979) *Biochem. J. 183*, 331−337.
Weisel, J. W., Nagaswami, C., Vilaire, G., & Bennett, J. S. (1992) *J. Biol. Chem. 267*, 16637−16643.
Williams, E. C., Janmey, P. A., Ferry, J. D., & Mosher, D. F. (1982) *J. Biol. Chem. 257*, 14973−14978.
Wolff, C., & Lai, C.-S. (1989) *Arch. Biochem. Biophys. 268*, 536−545.
Wolff, C. E., & Lai, C.-S. (1990) *Biochemistry 29*, 3354−3361.
Yamada, K. M., Kennedy, D. W., Kimata, K., & Pratt, R. M. (1980) *J. Biol. Chem. 255*, 6055−6063.
Yayon, A., Klagsbrun, M., Esko, Y. D., Leder, P., & Ornitz, D. M. (1991) *Cell 64*, 841−848.

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