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Interaction of Fibronectin with Heparin in Model Extracellular Matrices: Role of Arginine Residues and Sulfate Groups†

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ABSTRACT: The interaction of heparin with the NH_2-terminal domain of human plasma fibronectin was studied by using matrix-driven translocation, an assay for the adhesion of extracellular macromolecules with cell or particle surfaces within artificial collagen matrices. Partial desulfation of heparin rendered it ineffective in competitively inhibiting the interaction of the fibronectin NH_2-terminal domain with heparin-coated particles, suggesting a role for sulfate groups of heparin in the interaction. Analysis of the fibronectin domain in terms of its primary structure, its proposed organization into "type I modules", and its hydrophilic and flexible segments led to the identification of several arginine-containing sites of potential interaction with the sulfate groups of heparin. Modification of increasing numbers of arginine side chains with 1,2-cyclohexanedione under mild conditions eventually led to decreases in translocation-promoting activity, and of heparin binding capacity as measured in a gel-shift assay, but the major portions of these functions were retained even when the four most accessible arginines (attributed to sites in and adjacent to the large loops of the type I modules) were modified. With the modification of additional arginines (attributed to sites in the small loops), both functions were lost. The peptide Gly-Arg-Gly, corresponding to a repeated determinant at the tips of two small loops, inhibited translocation, but arginine alone did not. Cleavage of the large loops by CNBr also led to loss of translocation-promoting activity. The correspondence between the molecular determinants of matrix-driven translocation and those previously found for mesenchymal morphogenesis indicates the utility of this system in the analysis of adhesive interactions of biological importance.

Adhesive interactions of cells with glycoproteins of the extracellular matrix mediate a wide variety of tissue morphogenetic processes (Hay, 1981; Trelstad, 1984; McClay & Ettensohn, 1987). A number of assays are available for the analysis of these interactions, ranging in complexity from physical measurements of solution-phase binding between purified extracellular components (Wood, 1960; Horwitz et al., 1985), studies of cell attachment to planar substrata (Akiyama & Yamada, 1985; Ruoslahti & Pierschbacher, 1986; Laterra et al., 1983) and hydrated matrices (Tomasek et al., 1982; Guidry & Grinnell, 1987), to characterization of morphogenetic alterations in whole embryos brought about by specific inhibitors (Naidet et al., 1987) or genetic modification (Löhler et al., 1984).

Recently we described a model system in which adhesive interactions between distinct regions of an artificial extracellular matrix caused the rapid, unidirectional transport of suspended cells or latex particles into previously unpopulated regions of the matrix (Newman et al., 1985). We have termed this effect "matrix-driven translocation" (MDT).[1] MDT provides an adhesion assay for cell surface–extracellular matrix interactions that is intermediate in complexity between assays using isolated molecular components and those using whole tissues or embryos. This system combines advantages that are not found together in other assays for adhesive interactions of tissue components: in common with the tissue-based systems, the experimental end point is morphological, but like the standard biochemical assays, the nature and concentrations of interacting components can be varied at will.

MDT has proved useful in the identification of a specific interaction between the NH_2-terminal heparin binding domain of fibronectin and heparin-like components of the cell or particle surface (Newman et al., 1987). This interaction [which is overshadowed by the stronger interaction of the COOH-terminal region of fibronectin with immobilized heparin in column binding assays (Benecky et al., 1988)] promotes MDT, whereas interactions with other sites on fibronectin do not (Newman et al., 1987). More specifically, the promotion of MDT is associated with fibronectin–heparin interactions that bring about a specific transition in the circular dichroic spectrum of the 29-kDa fibronectin NH_2-terminal domain (FnNTD) (Khan et al., 1988). This has permitted discrimination among interactions such as those between fibronectin and heparin or dextran sulfate, which appear similar in affinity chromatography studies (Ruoslahti et al., 1979).

The biological relevance of the interactions assayed by MDT was suggested in recent studies of in vitro morphogenesis of embryonic limb bud mesenchymal cells (Frenz et al., 1989a,b). It was found that a monoclonal antibody directed against the FnNTD which was previously found to inhibit MDT (Newman

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[1] Abbreviations: MDT, matrix-driven translocation; FnNTD, 29-kDa fibronectin NH_2-terminal domain; DHCH-arginine, N^7,N^8-(1,2-dihydrocyclohex-1,2-ylene)-L-arginine.

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et al., 1987), and oligopeptides corresponding to a repeated motif in the same domain, disrupted mesenchymal morphogenesis, whereas monoclonal antibodies directed against other fibronectin domains, or closely related control oligopeptides, had no antimorphogenetic effect (Frenz et al., 1989b). Similarly, removal of heparin-like molecules from the mesenchymal cell surface, which had been found to render these cells refractory to MDT (Newman et al., 1987), also disrupted their morphogenesis (Frenz et al., 1989b).

The studies reported here were conducted to better define the fibronectin–heparin interaction that promotes MDT so as to gain more insight into related effects that may occur in living tissues. We have made use of the fact that free heparin interferes with the MDT-promoting interaction between the cell or particle surface and the FnNTD (Newman et al., 1987). By performing the assay in the presence of chemically modified heparins, we have now determined that sulfate groups of heparin are involved in this interaction. By modifying the FnNTD with 1,2-cyclohexanedione, we have found that a small number of the protein's arginines are also important in the interaction. Examination of the amino acid sequence of the FnNTD in relation to its proposed arrangement into disulfide-bonded loops and to its hydrophilicity profile suggests that the arginine side chains most critical for the interaction with heparin may lie in the small loops of the fibronectin type I modules of the FnNTD. The presence in the MDT assay of >70 μg/mL of the tripeptide Gly-Arg-Gly, which corresponds to a repeated surface motif in these small loops, was found to inhibit the interaction with high specifity. Because the same oligopeptide inhibited fibronectin-dependent mesenchymal morphogenesis in vitro (Frenz et al., 1989b), these results support the usefulness of the MDT assay in the analysis of biologically relevant cell–matrix interactions.

Experimental Procedures

Materials. Human plasma fibronectin was obtained from the New York Blood Center, exchanged into 0.2 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.2 (phosphate-buffered saline), on a Sephadex G-25 column, and used without further purification. The FnNTD was prepared by digesting the protein with tosylphenylalanyl chloromethyl ketone–trypsin (Worthington) followed by DEAE–cellulose chromatography as described (Hayashi & Yamada, 1983). The purity of the fragment was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and amino acid analysis. Beef lung heparin was purchased from Sigma (St. Louis, MO). Type I collagen was prepared by acid extraction from tail tendons of young adult rats (Elsdale & Bard, 1972) and dialyzed into one-tenth strength Ham's F-12 medium without bicarbonate (Gibco, Grand Island, NY). Oligopeptides were generously provided by Dr. Steven Akiyama, NIH (Gly-Arg-Gly-Asp; Gly-Arg-Gly-Asp-Ser; Arg-Gly-Asp-Ser), or custom-synthesized by Peninsula Laboratories (Belmont, CA) (Gly-Arg-Gly). In all cases, peptides were purified by reverse-phase high-performance liquid chromatography.

Matrix-Driven Translocation Assay. These assays were performed as previously described (Newman et al., 1985, 1987). Briefly, "primary gels" were constructed by suspending polystyrene latex beads (6 μm, Polysciences, Warrington, PA) in a soluble collagen solution (1.7 mg/mL) which was simultaneously adjusted to physiological pH and ionic strength. The final concentration of beads was 5×10^6/mL. "Secondary gels" consisted of an identical collagen solution with or without fibronectin (12.5 μg/mL), the FnNTD (5 μg/mL), or a modified version thereof (5–25 μg/mL). Drops of primary and secondary gel were placed contiguously on a horizontal plastic petri dish, and the subsequent movement of beads and their surrounding matrix across the original interface was recorded. MDT competitive inhibition assays were performed as previously described (Newman et al., 1987) by adding various heparin-related molecules to secondary gels along with fibronectin or the FnNTD, or by incubating beads with fibronectin-related or control peptides (50–200 μg/mL) for 30 min before constructing primary gels with the incubation mixtures. Gels were illuminated from below with a ring light and photographed through a dissecting microscope, as described (Newman et al., 1985).

Chemical Modification of Heparins. Solvolytic N-desulfation of heparin was carried out by treatment of its pyridinium salt with dimethyl sulfoxide containing 5% methanol for 1.5 h at 50 °C, as described (Inoue & Nagasawa, 1976). Desulfation by this method occurs without depolymerization of the heparin molecule. In particular, resulfated heparin had a virtually identical gel filtration profile with intact heparin (Inoue & Nagasawa, 1976). The extent of desulfation was determined to be 30% by elemental analysis of sulfur and nitrogen of treated and starting materials (Schwarzkopf Microanalytical Laboratory, Woodside, NY). This corresponds to approximately 75% removal of N-linked sulfates, since about 60% of the sulfates in heparin are O-linked (Rosenberg et al., 1978). Heparin methyl ester ("carboxymethylated heparin") was prepared by the diazomethane method of Danishefsky and Siskovic (1972). Chemical analysis of the modified heparin (Galbraith Laboratories, Knoxville, TN) showed that it contained 7.9% methoxyl, indicating that esterification was essentially complete.

Modification of FnNTD Arginine Residues. The arginine guanidino groups of the FnNTD were reversibly modified with 1,2-cyclohexanedione (Sigma) as described (Patthy & Smith, 1975). Briefly, the protein (1–2 mg) and an equal amount of 1,2-cyclohexanedione were separately dissolved in 1 mL of 0.2 M sodium borate buffer, pH 8.0. The solutions were warmed to 35 °C and mixed. The reaction was continued at the same temperature in the dark. Aliquots were taken after 0, 30, 60, 90, 120, and 180 min, and the reaction was stopped by neutralizing with an equal volume of 30% acetic acid. Reaction mixtures containing the modified fragment were dialyzed extensively against phosphate-buffered saline. Amino acid analysis was performed on each sample to determine the extent of arginine modification (Roosedorp et al., 1977).

CNBr Cleavage of FnNTD. Cyanogen bromide solution (100 mg/mL) in 70% formic acid was added to a solution of the FnNTD (0.5 mg/mL in 70% formic acid) to give a final molar ratio of 1:4 of total methionines to CNBr. The sample was incubated in the dark for 24 h with gentle stirring (Gross, 1967). Excess CNBr was removed by passing the reaction mixture through a Sephadex G-25 column.

"Gel-Shift" Assay for FnNTD–Heparin Binding. The FnNTD was electrophoresed under native conditions on 7% polyacrylamide minislab gels. The acrylamide:methylenebis(acrylamide) ratio was 30:1, and the gel buffer and running buffer contained 2 mM EDTA, 3 M urea, and 10 mM triethanolamine (TEA), pH 7.5. Protein (5 μg in 10 μL of phosphate-buffered saline) was added to an equal volume of 2× running buffer containing bromphenol blue before being loaded on the gel. Gels were run for 25–30 min and stained with 0.25% Coomassie Brilliant Blue in 50% methanol/10% acetic acid. Incubations of native or modified FnNTD with unmodified or modified heparins were performed at a heparin:protein ratio of 10:1 (w/w) in phosphate-buffered saline,

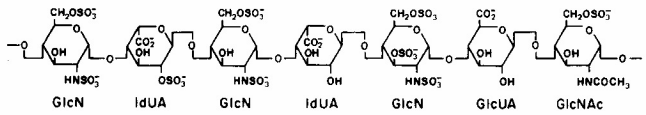

FIGURE 1: General structural diagram of heparin, indicating typical disaccharides and representative positions of sulfate and carboxylate groups. Abbreviations: GlcN, glucosamine; GlcNAc, acetylglucosamine; GlcUA, glucuronic acid; IdUA, iduronic acid. The specific sequence of saccharide units shown is for illustrative purposes only. (Adapted, with modifications, from Lindahl et al., 1977.)

before being mixed with sample buffer and being loaded on gel.

Computational Analysis of Protein Structure. The following analyses were performed by using programs supplied with the PC gene software package (Intelligenetics, Mountain View, CA): hydrophilicity index [ANTIGEN program of Hopp and Woods (1981)]; hydropathy index [SOAP program of Kyte and Doolittle (1982)]; position and sequence of flexible segments [FLEXPRO program of Karplus and Schulz (1985)]; secondary structure prediction by the method of Garnier et al. (1978).

RESULTS

Role of Heparin Determinants in MDT. Morphogenetic tissue interactions often involve cell surface heparan sulfate, a heterogeneous family of proteoglycans, the polysaccharide portion of which has a repeating disaccharide unit of glucosamine-hexuronic acid (Ruoslahti, 1988). Heparin is a distinct family of proteoglycans with the same general disaccharide repeat as heparan sulfate but a more restricted tissue distribution. Heparin is the more highly sulfated of the two glycosaminoglycans, but because of its similarity to certain sulfate-rich domains often found in heparan sulfate, it is commonly used as a functional analogue for the latter in studies of cell adhesive interactions (Gallagher, 1987). The presence of unmodified heparin in the fibronectin-containing gel was previously found to inhibit MDT (Newman et al., 1987). A generalized structural diagram of heparin is shown in Figure 1.

Specific removal or modification of anionic groups of heparin was carried out in order to determine their role in the MDT-promoting interaction of the glycosaminoglycan with the FnNTD. Approximately two-thirds of the N-linked sulfates in a commercial preparation of heparin were removed by treatment with dimethyl sulfoxide (Inoue & Nagasawa, 1976). This partially N-desulfated heparin was not inhibitory to MDT when added to the secondary (fibronectin-containing) gel, even at concentrations as high as 10 μg/mL, 5–10 times the concentration at which intact heparin was completely inhibitory (Figure 2).

Carboxyl groups of the uronic acid residues in the repeating disaccharide of heparin (Figure 1) were completely blocked by methyl esterification (Danishefsky & Siskovic, 1972). In contrast to the N-desulfated heparin, carboxymethylated heparin was as effective as intact heparin when used as a competitive inhibitor in the MDT assay (Figure 2).

Role of FnNTD Arginines in Promoting MDT. The amino acid sequence of the human FnNTD has been determined by Kornblihtt et al. (1985) on the basis of cDNA sequencing. We computed the hydrophilicity profile of the FnNTD using the method of Hopp and Woods (1981) (Figure 3). This analysis predicts a number of sites on the surface of the protein that can potentially interact with charged ligands. Comparison with the protein's sequence precludes a number of these sites for heparin binding because of their negative charge. The site predicted to be most hydrophilic contains two lysine residues

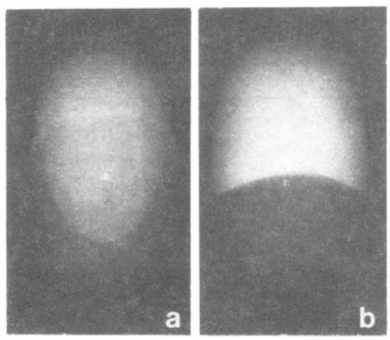

FIGURE 2: Competitive inhibition of MDT by chemically modified heparins. In (a) and (b), primary gels contained heparin-coated polystyrene latex beads. Secondary gels contained 12.5 μg/mL fibronectin in addition to 10 μg/mL N-desulfated (DS) heparin (a) or 2 μg/mL carboxymethylated (CM) heparin (b). Translocation is evident in panel a, but not panel b. Photographs are representative of results of at least three trials with each modified heparin.

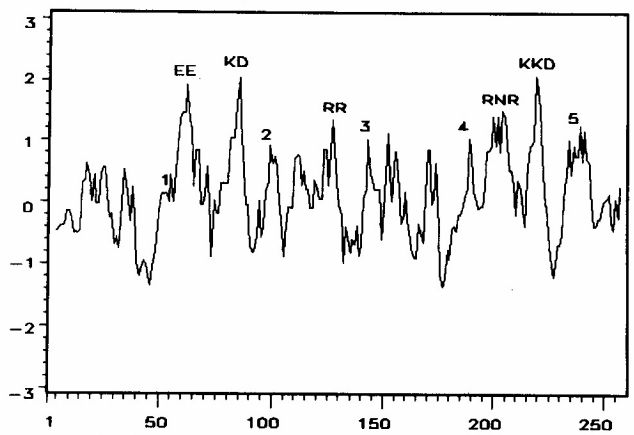

FIGURE 3: Hydrophilicity profile of the FnNTD. Hydrophilicity at each position was computed with the ANTIGEN program (Hopp & Woods, 1981) using an average group length of six amino acids. Protein sequence data based on Kornblihtt et al. (1985). Letters above peaks indicate charged or polar amino acid residues at corresponding positions. (Single-letter amino acid code: D, aspartic acid; E, glutamic acid; K, lysine; N, asparagine; R, arginine.) Numbers above peaks indicate tips of small loops of corresponding type I modules (see Figure 5).

(positions 217–218), but an adjacent aspartic acid residue makes this site a poor candidate for interaction with heparin. This leaves a double arginine at positions 124–125, followed by a nonbasic residue (Pro_{126}) and a basic one (His_{127}), which represents a consensus determinant for heparin binding (Cardin et al., 1989), an Arg-Asn-Arg at positions 197–199, and a number of solitary arginines and lysines throughout the sequence, as potential sites of interaction with heparin.

We covalently modified arginine residues in the FnNTD with 1,2-cyclohexanedione (Patthy & Smith, 1975) and used these modified proteins in the MDT assay. The modification method uses mild conditions, is highly specific for arginines, and leads to the formation of a single product, N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine (DHCH-arginine), which under the conditions of the MDT assay retains the positive charge of the guanidino group (Patthy & Smith, 1975). Preparations of the domain with an average of 0, 3.2, 4.3, 4.7, 7.4, and 8.0 modified arginines out of a total of 18 per molecule were obtained. MDT-promoting activity of the domain was largely spared by modification of an average of up to four arginine side chains per protein molecule, but was reduced to about 24% of control values when approximately five arginines per molecule were modified. Modification of two additional arginine residues per molecule completely eliminated MDT-promoting activity (Table I).

Table I: Effect of Various Chemical Modifications of FnNTD on Its Capacity To Promote MDT

FnNTD prepn	distance translocated[a]	(%)
arginines modified		
0	2.5 ± 0.03	100
3.2	2.0 ± 0.03§	80
4.3	1.8 ± 0.17	72
4.7	0.6 ± 0.08‖	24
7.4	0	0
8.0	0	0
CNBr cleaved	<0.25	<10

[a] Values given are the mean ± SEM of three experiments for each FnNTD preparation. An entry marked by (§) or (‖) is statistically different from the entry directly above it by the Student's t test, with §$p < 0.01$ or ‖$p < 0.05$.

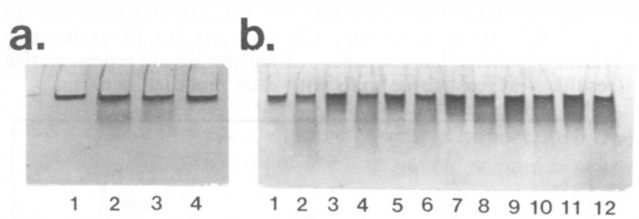

FIGURE 4: Gel-shift assay for binding of FnNTD to heparin. In (a), unmodified FnNTD was electrophoresed alone (lane 1) or preincubated with unmodified heparin (lane 2), carboxymethylated heparin (lane 3), or N-desulfated heparin (lane 4). In (b), electrophoresis was performed of unmodified FnNTD without (lane 1) or with (lane 2) preincubation with heparin, and preparations of FnNTD containing an average of 3.2 (lanes 3 and 4), 4.3 (lanes 5 and 6), 4.7 (lanes 7 and 8), 7.4 (lanes 9 and 10), and 8.0 (lanes 11 and 12) DHCH-arginines were electrophoresed without (odd lanes) or with (even lanes) preincubation with heparin.

The concentrations of modified FnNTDs used in these experiments were within 2–3-fold of the minimum required for a maximal response with the unmodified fragment. Therefore, the average numbers of modified arginines given in Table I are representative of the active protein species in the assay.

Native Polyacrylamide Gel Electrophoresis of Fibronectin in the Presence of Heparin. A qualitative "gel-shift" assay was devised to illustrate the binding of the FnNTD to heparin and changes in this interaction when either the glycosaminoglycan or the protein was modified. Under the conditions used, the FnNTD is near its isoelectric point (theoretical value, 7.4) and migrates only a short distance into the top of the gel (Figure 4a, lane 1). When preincubated with excess heparin, however, a portion of the protein binds to the polysaccharide, and migrates into the gel as a broad band (Figure 4a, lane 2). The amount of FnNTD that was shifted in mobility varied between preparations, possibly because of the strong tendency of the protein to aggregate at physiological pH (M.Y.K. and N.S.J., unpublished observations), but it was consistent within a given experiment. While the assay was therefore not readily applicable to quantitation of heparin binding, it provided a graphic means of detecting changes in binding upon modification of either of the two components.

Incubation of the FnNTD with carboxymethylated heparin led to a shift comparable to that brought about by intact heparin (Figure 4a, lane 3), but incubation with N-desulfated heparin led to no detectable change in mobility (Figure 4a, lane 4), indicating that the mobility shift was due to sulfate-dependent binding. When the arginine-modified FnNTDs used in the MDT experiments were incubated with intact heparin, mobility shifts were detected for modified preparations containing up to 4.7 equiv of modified arginine (Figure 4b, lanes 3–8). However, the most extensively modified of these active preparations (which was efficacious in the MDT experiments to only a minor extent; Table I) exhibited anomalous migration on the gel even in the absence of heparin (Figure 4b, lane 7). In preparations of modified FnNTD containing averages of 7.4 and 8.0 modified arginine residues, behavior on the native gels was highly anomalous, and there was no additional mobility shift brought about by incubation with heparin (Figure 4b, lanes 9–12).

Location of Arginine-Containing Sites Relative to Structural Organization of the FnNTD. The FnNTD consists of five "fibronectin fingers" or type I modules. Each of these modules is a disulfide-bonded structure consisting of a large and a small loop (Peterson et al., 1983). By fitting the amino acid sequence of the FnNTD to the pattern of type I modules, we located the arginine-containing sites of the protein in relation to these structural features (Figure 5). The arginine-containing sites predicted by the hydrophilicity profile to be most exposed to solvent (Figure 3), namely, the Arg-Asn-Arg at positions 197–199 and the Arg-Arg at positions 124–125, are located at the junction between the fourth and fifth type I module, and near the tip of the third large loop, respectively (Figure 5). These would presumably be the first four arginines to become modified by 1,2-cyclohexanedione. Interestingly, 5 of the remaining 14 arginines are located at or near the tips of the small loops, where they are invariably adjacent to the highly flexible residues glycine and serine (Figure 5). The second and fifth small loops contain the identical sequence, Gly-Arg-Gly, at their most distal sites, and the only small loop without an arginine, the third, has the sequence Gly-Lys-Gly in the analogous position (Figure 5). These are among the sites predicted to be most exposed to solvent by the hydrophilicity profile (Figure 3) and constitute major peaks of chain flexibility in the sequence, as calculated by the method of Karplus and Schulz (1985). Indeed, four of the small loop tips are among the eight most flexible segments of the protein [B(norm) $\geq$ 1.078].

Effect of CNBr Cleavage of the FnNTD on Its MDT-Promoting Activity. With the exception of one site five residues from the amino-terminal end, all the methionines of the FnNTD are located in the central three large loops (Figure 5, arrows). Cleavage of the protein with cyanogen bromide would release a pentapeptide with no positively charged residues, and create only single cuts in loops 2, 3, and 4. (The two methionines in loop 4 are adjacent to each other.) The integrity of the small loops and their linkage to one another would be unaffected, but their spatial relationships would be less constrained. We found that CNBr cleavage virtually completely destroyed the MDT-promoting activity of the FnNTD (Table I).

Role of the Small Loops. The gel-shift assay (Figure 4) indicated that the FnNTD with its four most exposed arginines modified, which we had previously found to have retained most of its MDT-promoting activity (Table I), also continued to bind heparin to an extent comparable to the unmodified protein. This result, in conjunction with the hydrophilicity profile of the fragment (Figure 3), directed our attention to the small loops of the FnNTD as potential sites of heparin binding and MDT-promoting activity. The second and fifth small loops of the FnNTD contain the tripeptide Gly-Arg-Gly at their tips, and these (along with the Gly-Ser-Gly-Arg in the fourth small loop) are predicted to be the next most highly exposed arginine-containing sites in the FnNTD. Because Gly-Arg-Gly is located in exposed, flexible segments of the protein, and was effective in disrupting the fibronectin-dependent condensation process in developing precartilage mesenchymal cells in vitro (Frenz et al., 1989b), we decided to test its effect on MDT in a competitive assay. We had

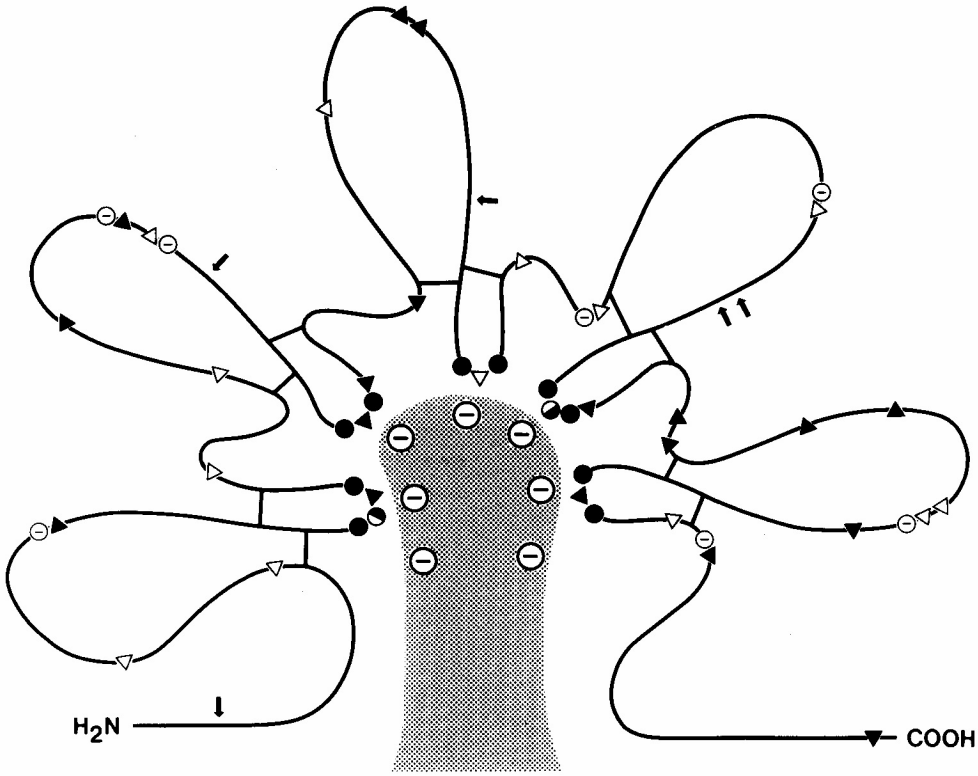

FIGURE 5: Schematic diagram of disulfide-bonded loop structure of the FnNTD showing sites of potential interaction with heparin. All positively charged residues in the domain are indicated by triangles [arginine (▲); lysine (△)]. All glycines (●) and serines (⊖) of the smaller loops are indicated, as well as negatively charged residues (⊖) whenever they are adjacent to arginines or lysines. Arrows mark the positions of cyanogen bromide cleavage sites at methionine residues. Primary structure of domain based on Kornblihtt et al. (1985). Loop organization based on Petersen et al. (1983). The stippled form represents the hypothesized relationship to the protein of one or more heparin-like molecules on the cell or bead surface.

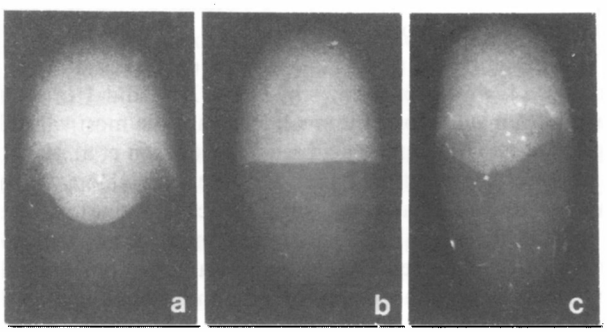

FIGURE 6: Competitive inhibition of MDT by Gly-Arg-Gly. In (a–c), primary gels contained heparin-coated beads, and secondary gels contained 12.5 μg/mL human plasma fibronectin. Primary gels also contained 100 μg/mL Arg-Gly-Asp-Ser (a), 70 μg/mL Gly-Arg-Gly (b), or 100 μg/mL arginine (c). Translocation is evident in panels a and c, but not in (b). Photographs are representative of results of at least three trials with each oligopeptide or amino acid.

Table II: Effect of Fibronectin-Related Oligopeptides and Arginine on the Capacity of Heparin-Coated Beads To Undergo MDT

peptide or amino acid	min concn for complete inhibn of MDT[a] (μg/mL)
Gly-Arg-Gly-Asp	25
Gly-Arg-Gly	70
Gly-Arg-Gly-Asp-Ser	>100[b]
Arg-Gly-Asp-Ser	≫100[c]
Arg	≫100[c]

[a] Heparin-coated 6-μm polystyrene latex beads were preincubated for 30 min with twice the designated concentrations of peptides; the beads in their incubation media were then mixed 1:1 with soluble collagen and used in the MDT assay. Peptide concentrations were increased by 5 or 10 μg/mL in successive experiments. Values given for the two peptides that were completely inhibitory represent the minimum concentration at which no translocation occurred in any of three replicate assays. [b] Partial inhibition at this concentration. [c] No inhibition at this concentration.

previously found that addition of fibronectin or the FnNTD to the primary (cell- or bead-containing) gel in the MDT assay completely inhibited the effect (Newman et al., 1985; D. Frenz and S. Newman unpublished results). When Gly-Arg-Gly was added to the primary gel at concentrations of 70 μg/mL or more, MDT did not occur (Figure 6; Table II). The oligopeptide Gly-Arg-Gly-Asp was somewhat more effective than Gly-Arg-Gly, and the integrin binding site related peptide Gly-Arg-Gly-Asp-Ser (Pierschbacher & Ruoslahti, 1987), much less so. The oligopeptide Arg-Gly-Asp-Ser, which is also disruptive of fibronectin's binding to integrin (Pierschbacher & Ruoslahti, 1987), was completely ineffective in blocking MDT (Table II). The free amino acid arginine, which contains the same N- and C-termini, and the same guanidino side chain, as Gly-Arg-Gly, was also completely ineffective in blocking MDT, even at molar concentrations more than 2.5-fold that of Gly-Arg-Gly (Figure 6, Table II).

Discussion

Earlier work on the MDT adhesion assay system addressed two distinct questions. These concerned the motive force for the coherent movement of cells or particles between the primary and secondary gels (Newman et al., 1985; Forgacs et al., 1989) and the molecular basis for the local interaction at the primary gel/secondary gel interface that initiates the movement (Newman et al., 1987; Khan et al., 1988). With respect to the first question, the most plausible force-generating mechanism appears to involve the thermodynamically spontaneous spreading or "wetting" behavior of two immiscible matrix droplets bounded by common substrata (Forgacs et al., 1989). Similar phenomena are observed in a variety of liquid (Torza & Mason, 1969) and in vitro tissue (Steinberg, 1978;

Armstrong, 1989) systems, and may be relevant to the understanding of morphogenetic events like gastrulation (Phillips & Davis, 1978) and tumor invasiveness (Straüli & Weiss, 1977).

Studies addressing the second question have identified the FnNTD as the relevant domain of fibronectin in promoting the interaction with heparin-like molecules on cell or latex particle surfaces (Newman et al., 1987; Khan et al., 1988), although the adjacent gelatin binding domain appears to enhance the effect in at least one fibronectin variant (Godfrey et al., 1989). Heparin induces a characteristic Ca^{2+}-suppressible conformational change in the FnNTD (as determined by circular dichroism) that is different from the structural alteration induced by dextran sulfate (Khan et al., 1988). The heparin-induced change appears to be related to the promotion of MDT by several criteria: MDT is also suppressible by Ca^{2+}; dextran sulfate coated beads do not undergo MDT nor does dextran sulfate share the capacity of heparin to competitively inhibit the effect (Newman et al., 1987). The present study provides further connections between these phenomena: partially N-desulfated but otherwise intact (Inoue & Nagasawa, 1976) heparin, which was previously found to have lost its capacity to induce the characteristic structural alteration in the FnNTD (Khan et al., 1988), is now seen to be ineffective in inhibiting MDT (Figure 2) or in causing a mobility shift of the FnNTD on a nondenaturing gel (Figure 4a).

The experiments reported here provide additional insight into the MDT-promoting interaction. Carboxymethylated heparin clearly interacts with the FnNTD, since it induces a structural change in the protein, but this change is qualitatively different from that induced by unmodified heparin (Khan et al., 1988). We have now shown that carboxymethylated heparin interferes with the MDT-promoting interaction (Figure 2) and causes a shift in the mobility of the protein on a nondenaturing gel (Figure 4a). This implies that while unblocked carboxyl groups may be essential for the MDT-associated conformational change induced by the glycosaminoglycan in the FnNTD (Khan et al., 1988), initial binding to the protein is mediated mainly by other moieties, such as sulfate groups, hence, the full retention of MDT inhibitory capacity by carboxymethylated heparin. We hypothesize that the *promotion* (in contrast to the competitive inhibition) of MDT requires the normal heparin-induced conformational change, and indeed latex beads coated with carboxymethylated heparin are not translocated by MDT (Jaikaria, 1989).

Heparin also interacts with collagen, and at concentrations as low as 5–10 μg/mL can affect the rate of fibrillogenesis (Wood, 1960), fibril morphology (Guidry & Grinnell, 1987), and the rheological properties of the resulting gels (McPherson et al., 1988). At the lowest concentration necessary for maximal inhibition of MDT (1 μg/mL), fully sulfated heparin did not affect the rate of collagen fibrillogenesis in our system (N.S.J., unpublished results), but we cannot exclude the possibility that inhibition of MDT by heparin is partly due to more subtle effects on collagen. On the other hand, our gel-shift assay demonstrates that the efficacy of a heparin species in inhibiting MDT corresponds directly to its ability to bind to the FnNTD. Because the mechanism of MDT may involve interaction of heparin-like cell or particle surface components with both collagen and fibronectin at the primary gel/secondary gel interface (Forgacs et al., 1989), free heparin could potentially interfere with the effect by binding to either protein.

The arginines of the small loops of the type I modules of the FnNTD contribute most critically to the capacity of this protein domain to promote MDT. After modification of the four most accessible arginines with 1,2-cyclohexanedione [presumably those in the second large loop, and at the junction between the fourth and fifth type I modules (Figures 3 and 5)], the FnNTD retained the bulk of its MDT-promoting activity. Heparin binding capacity in the gel-shift assay was also retained under these conditions (Figure 4b, lanes 5 and 6), but activity was largely lost with the modification of as little as 0.5 additional equiv of arginine [most likely representing a distribution of sites at or near the tips of the small loops (Figures 3 and 5)] and was completely gone after modification of a total of seven arginines (Table I).

The loss of heparin binding that occurred when between five and seven arginines were modified was not attributable to increased aggregation of the protein, since this change was accompanied by *increased* mobility of the modified protein on the nondenaturing gel (Figure 4b, lanes 7–12), and while it is possible that modifications beyond an average of four arginines affected the FnNTD's function by virtue of a change in the protein's conformation rather than in the binding capacity of the additionally modified arginine side chains, we think that this is unlikely since charged side chains are not generally major determinants of a protein's folded state (Dill, 1990).

The effectiveness and specificity of the oligopeptide Gly-Arg-Gly in inhibiting MDT (Figure 6; Table II) further suggest that the ligand binding capacity of the small loops is of major importance in the effect. However, we were unable to inhibit binding of the FnNTD to heparin–Sepharose with up to 400 μg/mL Gly-Arg-Gly (M.Y.K. and S.A.N., unpublished results), suggesting that binding to immobilized heparin in the column assay may be dominated by sites located outside the small loops.

When compared among all known examples of type I modules (12 in fibronectin, 1 in factor XII, and 1 in tissue plasminogen activator), the small loops are the most variable regions in terms of amino acid sequence (Baron et al., 1990). It is therefore significant that the five small loops in the FnNTD all contain arginine (or in one case, lysine) residues at or near their tips in a local environment of glycines and serines. Arginine residues in particular have been widely confirmed to serve a general role as anion binding sites in proteins (Riordan, 1979). The small loops of the type I modules are ideal for presenting the guanidino side chains of arginines to negatively charged ligands such as the sulfate groups of heparin. A recent structural analysis of the seventh type I module of human fibronectin by nuclear Overhauser enhancement spectroscopy shows the small loop to be the most flexible portion of the module (Baron et al., 1990). These loops are 11–12 residues long, consist largely of nonhelical, nonextended structure, and in general fulfill the criteria of Ω loops, which are almost invariably situated at the protein surface (Leszczynski & Rose, 1986). Analysis of the protein's hydropathy profile confirms their assignment to the surface. The small loops may act in a cooperative fashion to create a high-specificity heparin binding site. The loss of MDT-promoting activity brought about by CNBr cleavage of the FnNTD (Table I) is consistent with the possibility that translocation requires a constrained spatial configuration of the small loops.

The utility of the MDT system in analyzing adhesive interactions of biological importance can be seen in the correspondence between the various fibronectin-related antibody and peptide reagents that disrupt both MDT (Newman et al., 1987; the present study) and mesenchymal morphogenesis

(Frenz et al., 1989a,b). It is possible that the collagen matrix in the MDT system provides a medium for the interaction of fibronectin or the FnNTD with heparin-like surface molecules that mimics the tissue environment. Structural changes induced in the protein by heparin that are detectable by circular dichroism (Khan et al., 1988), polarization of fluorescence (Khan et al., 1990), and solvent-perturbed fluorescence (Khan et al., 1990) may be manifested as a morphogenetic adhesive response in this assay system. The validity of such correspondences can be continually tested by comparing results obtained in the easily manipulated MDT system with those obtained in a variety of protein–ligand interaction assays and tissue-based experimental systems.

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