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Interaction of the NH_2-terminal Domain of Fibronectin with Heparin

ROLE OF THE Ω-LOOPS OF THE TYPE I MODULES*

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Determinants of the interaction of the 29-kDa NH_2-terminal domain of fibronectin with heparin were explored by analysis of normal and mutant recombinant NH_2-terminal fibronectin fragments produced in an insect cell *Baculovirus* host vector system. A genomic/cDNA clone was constructed that specified a secretable human fibronectin NH_2 fragment. With the use of site-directed mutagenesis a set of 29 kDa fragments was obtained that contained glycine or glutamic acid residues in place of basic residues at various candidate sites for heparin binding in the five type I modules that make up the domain. The recombinant fragment containing the wild type sequence had a nearly normal circular dichroic spectra and a melting profile, as assayed by loss of ellipticity at 228 nm, that was indistinguishable from that of the native fragment obtained by trypsinization of plasma fibronectin. A substantial proportion of the wild type recombinant fragment bound to heparin-Sepharose, where it was eluted at the same NaCl concentration as the native fragment. The wild type fragment was capable of promoting matrix-driven translocation, a morphogenetic effect in artificial extracellular matrices that depends on the interaction of the fibronectin NH_2 terminus with heparin-like molecules on the surfaces of particles. Mutant fragments in which arginines predicted to be most exposed in the folded fragment were converted to glycines retained the same affinity for heparin as the wild type fragment. In contrast, a mutant fragment in which the single basic residue (Arg^{99}) in the minor loop ("Ω-loop") of the second type I module was converted to a glycine had an essentially normal melting profile but exhibited no binding to heparin and failed to promote matrix-driven translocation. A mutant fragment in which the single basic residue (Arg^{52}) of the first type I module was converted to a glycine also completely lacked heparin binding activity, but one in which the single basic residue (Arg^{191}) the fourth type I module was converted to a glycine retained the ability to bind heparin. A mutant fragment in which the single basic residue (Lys^{143}) in the Ω-loop of the third type I module was converted to a glutamic acid lacked heparin binding activity but had a CD spectrum similar to the heparin-liganded native protein and was capable of promoting matrix-driven translocation. The results indicate that multiple residues in the Ω-loops of the fibronectin NH_2-terminal domain participate in its interactions with heparin. In addition, the conformation of one of the nonbinding mutants may mimic the heparin-induced structural alteration in this fibronectin domain required for certain morphogenetic events.

Fibronectin, an adhesive glycoprotein of the blood plasma and extracellular matrix, contains two distinct sites with affinity for the glycosaminoglycan heparin (see Refs. 1 and 2 for reviews). One of these sites, Hep2, near the carboxyl-terminal end of the protein comprises one or more disulfide-lacking type III fibronectin modules (3, 4) and may mediate cell adhesion and neurite outgrowth (5, 6). The other heparin-binding site, Hep1, is coincident with fibronectin's compact, trypsin-resistant, 29-kDa amino-terminal domain. This domain, which consists of five disulfide-containing type I fibronectin modules ("fibronectin fingers"), apart from its heparin-binding capability, is an important site of binding to fibrin (7–10), thrombospondin (11), tumor necrosis factor-α (12), surface proteins of *Staphylococcus aureus* (13, 14), as well as an essential component of fibronectin's ability to assemble into an extracellular matrix (15, 16).

Previous studies have suggested that the heparin binding capability of the 29-kDa NH_2-terminal domain of fibronectin (FnNTD)[1] also helps mediate the cellular condensation process that occurs at sites of incipient chondrogenesis in the precartilage mesenchyme of the developing vertebrate limb (17, 18). In particular, the formation of condensations in high density cultures of precartilage mesenchyme was inhibited by a monoclonal antibody directed against the FnNTD and by tri- or tetrapeptides containing Gly-Arg-Gly (19), a conserved, repeated motif of the FnNTD (2) that represents a putative determinant of the heparin binding capacity of this domain (20). Moreover, cell-sized polystyrene latex beads coated with heparin, when mixed with limb mesenchymal cells, accumulated at fibronectin-rich sites of cellular condensation during early chondrogenesis *in vitro*, but beads coated with dextran sulfate did not. This redistribution of heparin-coated beads was also inhibited by the anti-FnNTD antibody and by Gly-Arg-Gly (21). In the condensing limb bud mesenchyme the presumed heparin-like ligand for the FnNTD is the cell surface heparan

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[1] The abbreviations used are: FnNTD, fibronectin 29-kDa NH_2-terminal domain; Wt, wild type; MDT, matrix-driven translocation; kbp, kilobase pair; TPCK, tosylphenylalanyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis.

sulfate-containing proteoglycan, syndecan, which, like fibronectin, is abundant at sites of precartilage condensation (22).

The morphogenetic potential of the interaction between the FnNTD and heparin-like molecules was also demonstrated in experiments that assayed for matrix-driven translocation (MDT), a morphological reorganization that occurs when an artificially constructed extracellular matrix containing heparin-coated polystyrene beads is placed adjacent to similar matrix containing fibronectin (23, 24). Under the conditions of the assay the FnNTD was the only domain of fibronectin both necessary and sufficient for MDT to occur (24). MDT did not occur when dextran sulfate-coated beads were substituted for heparin-coated beads (23) or when tri- or tetrapeptides containing Gly-Arg-Gly were used as competitors of the bead-fibronectin interaction (20) and was greatly reduced when FnNTDs were used in which more than four arginines were blocked by the chemical agent 1,2-cyclohexanedione (20). This degree of chemical modification also essentially eliminated the heparin binding capacity of this fibronectin domain (20). It was concluded from these studies that the arginines (and possibly a lysine) in the highly flexible minor loops of the FnNTD type I modules (25) were likely sites of interaction with heparin. Because none of the individual minor loops contains a canonical heparin binding site (26) and limited internal cleavage of the major loops by cyanogen bromide destroyed the MDT promoting activity of the FnNTD (20), it was further suggested that cooperation among multiple minor loops is required for the interaction of this fibronectin domain with heparin (20). (Following Leszczynski and Rose (27), we refer to these small, compact loops as "Ω-loops").

In the present study we have tested these inferences directly by producing recombinant FnNTDs with site-directed modifications that eliminated one or more basic side chains at sites predicted to be the most highly exposed in the human FnNTD and at the somewhat less exposed sites in the Ω-loops of modules I-1, I-2, I-3, and I-4. We found, as predicted from the earlier chemical modification studies, that elimination of arginines outside the Ω-loops had no effect on heparin binding of the FnNTD, whereas modifications of the single basic residues in the Ω-loops of I-1, I-2, or I-3 eliminated all of the fragment's heparin binding activity. The mutant fragments containing either or both of the alterations in the Ω-loops of I-2 and I-3 were tested as well for ability to promote the MDT *in vitro* morphogenetic effect and, by CD, for retention of native conformation in the absence and the presence of heparin. Two of these mutant fragments were incapable of promoting MDT, as expected. However, the fragment mutated in the Ω-loop of I-3, in which a positively charged side chain was replaced by a negatively charged one, retained the ability to promote MDT, despite the virtual elimination of its heparin binding activity. The CD measurements suggested that in this case the mutation may have thrown the fragment into its "morphogenetically active" conformation. The results indicate that basic residues in the Ω-loops of the FnNTD are decisive in heparin binding and some morphogenetic effects mediated by this domain and suggest that loop-loop interactions may play a role in these processes.

EXPERIMENTAL PROCEDURES

Materials—Human plasma fibronectin was obtained from the New York Blood Center and exchanged into phosphate-buffered saline (0.02 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl) and used directly or after being digested with tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) as described (28). Purified beef lung heparin was generously provided by the late Dr. Isidore Danishefsky. Rat monoclonal antibody 304, reactive against the FnNTD (24), was a gift from Dr. Steven Akiyama (Laboratory of Developmental Biology, NIDR). cDNA clone pFH6, encoding the human FnNTD and a portion of the adjacent collagen-binding domain (29), was a gift from Dr. Francisco Baralle (International Center for Genetic Engineering and Biotechnology, Trieste, Italy). Genomic DNA clone pgHF3.7, encoding the human fibronectin promoter, secretory signal sequence, and the initial portion of the FnNTD (30), was a gift from Dr. Douglas Dean (Washington University, St. Louis, MO). Oligonucleotides for sequencing and site-directed mutagenesis were synthesized in the Department of Biochemistry and Molecular Biology (New York Medical College) or purchased from Gene Link, Inc. (Thornwood, NY). Sf21 insect cells were obtained from Invitrogen (San Diego, CA) and maintained in shaker flasks in serum-free insect cell culture medium (Life Technologies, Inc.). Type I collagen was obtained by acetic acid extraction of tail tendons of young adult rats and kept frozen at −20 °C as described (23). The collagen was dialyzed into acidified 1:10 strength Ham's F-12 medium without bicarbonate (Flow Laboratories, McLean, VA) before being used in the MDT assay (23, 24).

Construction of a Genomic/cDNA Clone Specifying Secretable 54-kDa Amino Terminus of Human Fibronectin—Clone pFNs54 was constructed from two pre-existing clones as follows. (i) Human fibronectin cDNA clone pFH6 (29) was digested with *Esp*I and *Bam*HI, yielding a 1.2-kbp fragment encoding the initial 54 kDa of the mature protein. (ii) Human genomic clone pgHF3.7 (30) containing the 5′ end of the fibronectin gene, including a small region of overlap with pFH6, was digested with *Pst*I and *Esp*I, yielding a 291-base pair fragment containing the complete signal sequence and a 3′ terminus at the same position as the 5′ terminus of the fragment generated from pFH6. The two fragments were ligated at their common *Esp*I site and cloned into the *Pst*I-*Bam*HI site of pUC18 (31), yielding the plasmid pFNs54, specifying a secretable form of the NH_2-terminal 54 kDa of the mature fibronectin protein.

Site-directed Mutagenesis of Fibronectin cDNA—The 1.5-kbp *Pst*I-*Bam*HI fibronectin cDNA fragment was excised from pFNs54 and used for single-stranded (Ref. 32; Sculptor kit version 2.1, Amersham Corp.) or double-stranded (Ref. 33; Chameleon kit, Stratagene, La Jolla, CA) site-directed mutagenesis. Mutagenic oligonucleotides were phosphorylated with T4 polynucleotide kinase and annealed with the target sequences. Seven different mutants were made; the corresponding mutagenic oligonucleotides used (with the mutated base underlined) and the predicted amino acid changes are as follows: M1, 5′-TCTCCCTC-CCCCAGCCCC-3′ for $Arg^{99} \rightarrow Gly^{99}$; M2, 5′-ATTCTCCTTCTCCAT-TACC-3′ for $Lys^{143} \rightarrow Glu^{143}$; in M1+2, the second of the above substitutions was made in a clone in which the first substitution had already been made, yielding a clone that encoded both Gly^{99} and Glu^{143}; M3, 5′-GGAGGAAGCGGAGGTTTTAACTGCG-3′ for $Arg^{52} \rightarrow Gly^{52}$; M4, 5′-GGTGACACCTGGGGGGGACCACATGAGACTGG-3′ for Arg^{124}-$Arg^{125} \rightarrow Gly^{124}$-$Gly^{125}$; M5, 5′-GGCAGCGGAGGCATCACTTGC-3′ for $Arg^{191} \rightarrow Gly^{191}$; M6, 5′-GCACTTCTGGAAATGGATGCAACGAT-CAGG-3′ for Arg^{197}-Asn^{198}-$Arg^{199} \rightarrow Gly^{197}$-$Asn^{198}$-$Gly^{199}$. The presence of the desired mutations was confirmed by the dideoxy sequencing method using the Sequenase sequencing kit (U. S. Biochemical Corp.) with appropriate primers.

Expression Cloning of Wild Type and Mutant FnNTDs in Insect Cells—The 1.5-kbp *Pst*I-*Bam*HI wild type and mutant fibronectin cDNA fragments were ligated into the polylinker region of the *Baculovirus* transfer vector pVL1392 (Pharmingen, Inc., San Diego, CA) in the same orientation as the polyhedrin protein promoter (34). Co-transfection of Sf21 insect cells was carried out using linearized *Baculovirus* DNA (Baculogold, Pharmingen) and recombinant fibronectin/pVL1392 DNA in calcium chloride solution. The transfected cells were grown in serum-free insect cell culture medium at 27 °C for 4 days, at which time the recombinant viruses were amplified to obtain a high titer stock (1×10^8 viral particles/ml). Subsequent transfections were done in both T-flask and shaker flask culture at a multiplicity of infection of 5–10. The infected cells were grown for 3 or more days, after which the supernatant was harvested, centrifuged at $5,000 \times g$ for 10 min, and cleared by an additional 10 min of centrifugation at $10,000 \times g$. Supernatants were analyzed on 12.5% SDS-PAGE gels (35) by Coomassie Blue staining or after electrotransfer to nitrocellulose by immunoblotting with monoclonal antibody 304 (24) and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody to confirm and estimate the level of expression of the FnNTDs.

Purification of Wild Type and Mutant FnNTDs—The cleared culture supernatants were dialyzed against 25 mM Tris-HCl, pH 7.6, 25 mM NaCl, 0.5 mM EDTA for 2 days, changing the buffer every 8 h. Protein concentrations were determined by the Bradford method (36) or, after purification of fibronectin fragments, by absorbance at 280 nm using a specific extinction coefficient of 12.8 (37). To generate the 29-kDa FnNTDs, the 54-kDa recombinant fibronectin NH_2-terminal fragments were digested with TPCK-treated trypsin at a fibronectin:trypsin ratio

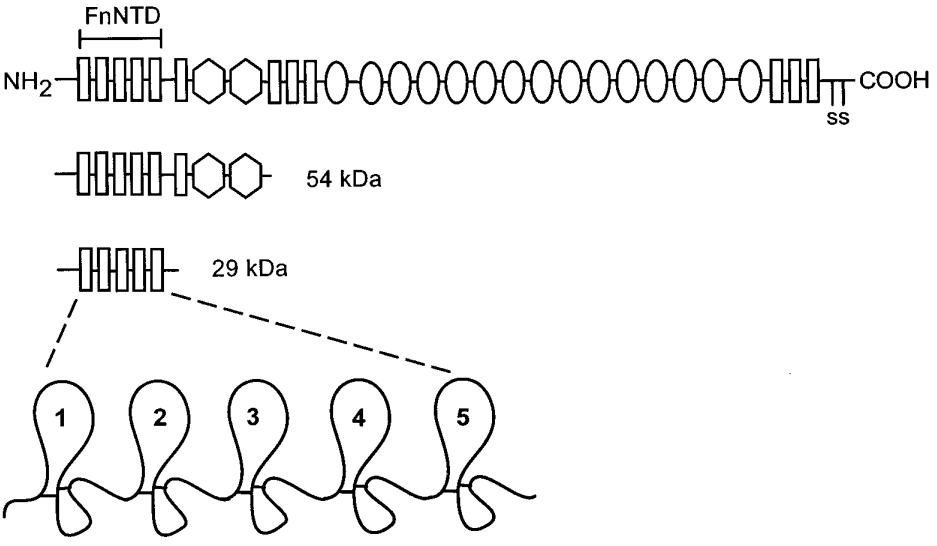

FIG. 1. **Schematic representation of modular organization of fibronectin and the fragments used in this study.** One chain of the dimeric protein is shown, with *rectangles* representing type I modules, *hexagons* representing type II modules, and *ovals* representing type III modules. The 54-kDa protein secreted from Sf21 insect cells under the direction of recombinant *Baculovirus* containing the ~1.5-kbp insert from pFNs54 comprises the first six type I and two type II modules from the NH_2 terminus of fibronectin. The 29-kDa FnNTD is generated by trypsinization of the intact native protein or the 54-kDa recombinant fragments. The peptide backbone of the five type I modules of the FnNTD is represented.

of 500:1 at 25 °C for 15 min, followed by addition of phenymethylsulfonyl fluoride to a final concentration of 1 mM (28). The digested protein was further dialyzed against 25 mM Tris-HCl, pH 7.0, 25 mM NaCl, 0.5 mM EDTA, 1 mM phenymethylsulfonyl fluoride. The dialyzed samples were passed through a DEAE-cellulose column equilibrated with the same buffer. The wild type recombinant FnNTD, like the native fragment obtained by digesting plasma fibronectin (28), was not bound and was recovered from the prewash. For some experiments the wild type fragment was further purified by affinity chromatography on heparin-Sepharose (see below). Mutant fragment M4 was eluted from DEAE-cellulose with 100 mM NaCl, M2 and M5 were eluted with 150 mM NaCl, and M1, M1+2, M3, and M6 were eluted with 200 mM NaCl. The mutant proteins were purified away from contaminating species by gel filtration chromatography using Sephacryl-100 followed, where required, by fast protein liquid chromatography with a Superose-12 column (Pharmacia Biotech Inc.). Protein samples used in analytical experiments were >95% pure by the criterion of relative intensity of the 29-kDa band to other species on Coomassie-stained SDS-PAGE gels.

Circular Dichroism—CD measurements in the far UV region (200–250 nm) were carried out on a Jasco model 500C spectropolarimeter equipped with a microcell holder, ADLAB-PC adapter, and ADAPT program (Interactive Microware, State College, PA). Spectra were obtained in phosphate-buffered saline at room temperature with a time constant of 16 s, a sensitivity of 0.1 millidegree/cm and a scan rate of 5 nm/min in a cuvette with an optical path length of 0.1 cm. Protein concentrations were in the range of 0.32–0.48 mg/ml. When heparin was present, the weight ratio of heparin to protein was 0.005–0.025, and the signal for heparin alone in the same buffer, though minimal, was corrected for. The data were collected at 0.2 nm intervals, and the average of 10 scans was reported. Melting curves were obtained by monitoring ellipticity at 228 nm while heating a jacketed cell of the same path length, 0.1 cm, at 1 °C/min. A mean residue molecular weight of 112 was used in the calculations of ellipticity. Secondary structures were estimated from CD data using the fixed reference method (CDESTIMA program) of Yang *et al.* (38). The ellipticity at each nm from 240 to 200 nm was used in the CDESTIMA analysis, as described (39). The reference spectra were pure component spectra extracted from CD analysis of proteins with structures previously determined by x-ray diffraction.

Heparin-Sepharose Chromatography of the FnNTDs—Heparin-Sepharose mini-columns (Pharmacia) were equilibrated with 10 mM sodium phosphate, pH 7.2, 50 mM NaCl. Each purified FnNTD (0.2 ml; 0.11–0.22 mg/ml) was loaded onto a column, and a gradient of 50–750 mM NaCl in the same buffer was applied at room temperature at a flow rate of 0.5 ml/min. The absorbance of the column effluent at 280 nm was monitored with a flow cell.

Matrix-driven Translocation—This assay was performed as described previously (23, 24). Briefly, "primary gels" were constructed by suspending polystyrene latex beads (6 μm, Polysciences, Warrington, PA; final concentration, 5×10^6/ml) in a cooled, soluble collagen solution (1.7–3.5 mg/ml), which was simultaneously adjusted to physiological pH and ionic strength with concentrated Ham's F-12 medium and sodium bicarbonate. "Secondary gels" consisted of an identical collagen solution with or without 6 μm/ml FnNTD or one of its mutant variants. Drops of primary and secondary gel were placed contiguously on a plastic Petri dish, and the subsequent movement, or lack thereof, of beads and surrounding matrix across the original interface was recorded. Because certain commercially fabricated polystyrenes are well established mimics of heparin in MDT (20) and other protein binding assays (40, 41), uncoated beads having this property were used in most of these experiments.

RESULTS

Expression of Wild Type and Mutant FnNTDs—The modular organization of fibronectin is shown schematically in Fig. 1, along with the relative positions of the fragments used in these studies. Sf21 insect cells infected with recombinant *Baculovirus* containing ~1.5-kbp human fibronectin genomic/cDNA inserts derived from pFNs54 (Fig. 2*A*) were grown in serum-free medium where they secreted protein fragments corresponding to the first 54 kDa of the mature fibronectin molecule. These fragments were digested with trypsin and purified to yield wild type and mutant FnNTDs (Fig. 2*B*). Wild type (Wt) recombinant FnNTD was produced using the parental insert from pFNs54 and contained a nucleotide sequence that encoded the protein sequence of the first five type I modules (I-1–5) of human fibronectin (42), plus I-6 and the protein's two type II modules (II-1 and II-2) (Fig. 1). Mutant 1 (M1) encoded an Arg → Gly substitution at the tip of the minor loop of module I-2 (amino acid 99 from the mature NH_2 terminus). M2 encoded a Lys → Glu substitution at the corresponding position in module I-3 (amino acid 143), and M1+2 encoded both of these substitutions. M3 and M5 encoded Arg → Gly substitutions near the tips of the minor loops of modules I-1 (amino acid 52) and I-4 (amino acid 191), respectively. M4 encoded a Gly-Gly substitution for the Arg-Arg in the large loop of module I-3 (amino acids 124 and 125), and M6 encoded Arg → Gly substitutions for both of the arginine residues flanking an asparagine residue in the protein strand connecting the small loop of module I-4 with the large loop of module I-5 (amino acids 197 and 199) (Fig. 3). Sequencing of several hundred nucleotides to either side of the mutant sites disclosed no changes other than the desired mutations.

Of all the tryptic fragments of fibronectin, the FnNTD is the only one that fails to bind to DEAE-cellulose, making its purification a one-step procedure (28). We found that Wt FnNTD behaved in this respect exactly like the native fragment prepared from plasma fibronectin but that all the mutant FnNTDs we studied bound to the DEAE-cellulose column and required other methods for their purification (see "Experimental Procedures"). Because the indicated mutations were designed to

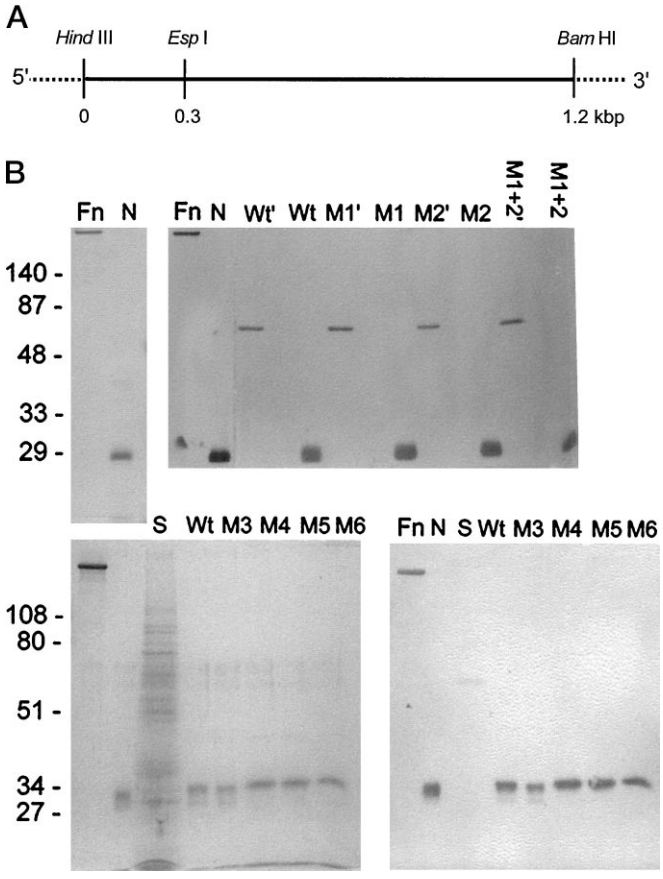

FIG. 2. **Production of wild type and recombinant FnNTDs in the Sf21 insect cell *Baculovirus* host vector system.** *A*, schematic representation of the genomic/cDNA clone pFNs54. The coding region for the human fibronectin secretory sequence, contained between the *Pst*I and *Esp*I sites, and the coding region for the first 54 kDa of the mature protein, contained between the *Esp*I and *Bam*HI sites, were derived from a genomic and a cDNA clone, respectively. The 1.5-kbp hybrid fragment was inserted into pUC18 for propagation; this construct is referred to as pFNs54. The fragment was inserted into bacteriophage M13 m8 or pWhitescript for site-directed mutagenesis and into *Baculovirus* transfer vector pVL1392 for protein production in insect cells. See "Experimental Procedures" for details. *B*, *upper left*, 12.5% SDS-PAGE gel containing intact fibronectin (*Fn*) and native FnNTD prepared by trypsinization of fibronectin (*N*); *upper right*, immunoblot, using monoclonal antibody 304 as primary antibody, of intact fibronectin (*Fn*), native FnNTD (*N*), partially purified *Baculovirus*-specified wild type and mutant 54-kDa fibronectin NH_2-terminal fragments (*Wt'*, *M1'*, *M2'*, and *M1+2'*, respectively), and purified wild type and mutant FnNTDs prepared by trysinization of recombinant products (*Wt*, *M1*, *M2*, and *M1+2*, respectively); *lower left*, 12.5% SDS-PAGE gel containing intact fibronectin (*Fn*), native FnNTD (*N*), supernatant of insect cell culture infected with wild type FnNTD recombinant *Baculovirus* (*S*), and purified wild type and mutant FnNTDs (*Wt*, *M3*, *M4*, *M5*, and *M6*, respectively); *lower right*, immunoblot using monoclonal antibody 304 as primary antibody of intact fibronectin (*Fn*), native FnNTD (*N*), culture supernatant (*S*), and purified *Baculovirus*-specified wild type and mutant FnNTDs (*Wt* and *M3–M6*, respectively).

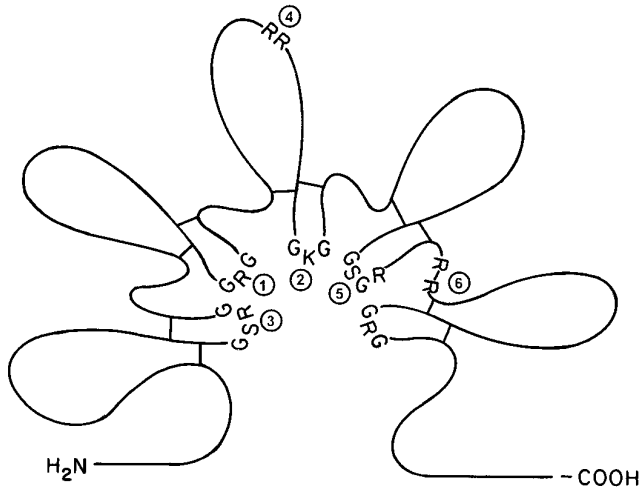

FIG. 3. **Wild type amino acid sequences at target sites of mutagenesis in the FnNTD.** Predicted amino acid sequence changes corresponding to *circled numbers* were: *1*, $Arg^{99} \rightarrow Gly^{99}$; *2*, $Lys^{143} \rightarrow Glu^{143}$; *3*, $Arg^{52} \rightarrow Gly^{52}$; *4*, Arg^{124}-$Arg^{125} \rightarrow Gly^{124}$-$Gly^{125}$; *5*, $Arg^{191} \rightarrow Gly^{191}$; and *6*, Arg^{197}-Asn^{198}-$Arg^{199} \rightarrow Gly^{197}$-$Asn^{198}$-$Gly^{199}$. Mutant M1+2 specified an FnNTD predicted to contain changes *1* and *2*.

alter the charge properties of surface domains of FnNTD, the altered DEAE-cellulose binding properties were not surprising.

Structural Characteristics of Recombinant FnNTD—The far UV CD spectrum of the Wt recombinant FnNTD was similar to that previously reported for the native 29-kDa NH_2-terminal fragment (43–45) (see below). In common with only a small number of other proteins, fibronectin and some of its fragments, notably the FnNTD, exhibit an unusual positive peak at ~230 nm that is shared by only a small number of other proteins (46–48). The mean residue ellipticity at 228 nm for the Wt fragment was 25–30 $\times 10^2$ deg cm^2 dmol^{-1}, which was comparable with the values for the native fragment obtained in previous studies (43–45, 49) and confirmed in the present study (not shown). That of M1 was 45–50 $\times 10^2$ deg cm^2 dmol^{-1} (see below). (Intact fibronectin, in contrast, has a maximum mean residue ellipticity of about 15 $\times 10^2$ deg cm^2 dmol^{-1} at 228 nm). The positive CD peak provides the basis for a sensitive assay for the structure of the FnNTD, because it is largely lost when the protein is denatured (49). The melting profile of the Wt FnNTD monitored at 228 nm was indistinguishable from that of the native fragment. The melting profile of M1 was essentially normal at elevated temperatures (Fig. 4).

For the thermal denaturation measurements the Wt fragment was purified away from heparin nonbinding (most likely conformationally abnormal) molecules by heparin-Sepharose chromatography (Fig. 5, see below) in addition to the standard DEAE-cellulose and gel filtration methods. This provided a normal standard of comparison for the analysis of the mutants. For example, whereas a similar affinity purification was not possible for M1, the similarity of its melting profile to that of the heparin-binding fraction of the Wt preparation suggests that its failure to bind heparin (Fig. 5, see below), was attributable more to the loss of a key surface group than to gross misfolding of the protein molecules.

Heparin Binding Properties of FnNTDs—Analytical affinity chromatography on heparin-Sepharose columns was performed for the native, wild type, and mutant FnNTDs (Fig. 5). More than 90% of the native fragment bound to the column in the low salt application buffer, and most of this was eluted in a peak centered at 300 mM NaCl. About 55% of the Wt recombinant fragment bound to the column, and this eluted at essentially the same salt concentration as the native fragment (Fig. 5). The nonbinding fraction of the Wt sample may have comprised conformationally abnormal FnNTD molecules but did not appear to represent proteins other than FnNTD in the preparation (Fig. 2*B*). The sites mutated in clones M4 and M6 represent the basic regions of highest predicted hydrophilicity in the FnNTD (20) and are thus the sites that would be predicted to mediate interaction of the domain with heparin (26). It was therefore of interest that substantial portions of the protein specified by these mutant clones bound to the heparin column and eluted at the same salt concentration as the native and Wt proteins. We interpret this as indicating that the loss of basic surface groups in M4 and M6 had little effect on the heparin affinity of the FnNTD but that there was a variable amount of

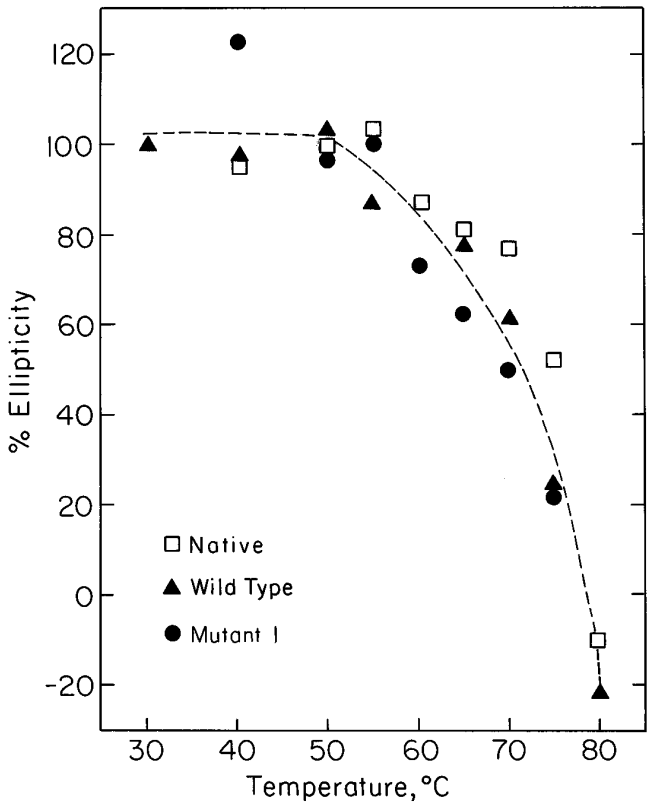

FIG. 4. **Melting profiles of FnNTDs monitored by circular dichroism.** Molar ellipticities at 228 nm were calculated for spectra of native (*squares*), wild type (*triangles*), and M1 (*circles*) proteins taken at temperatures between 30 and 80 °C. Data are expressed as percentages of native values at room temperature.

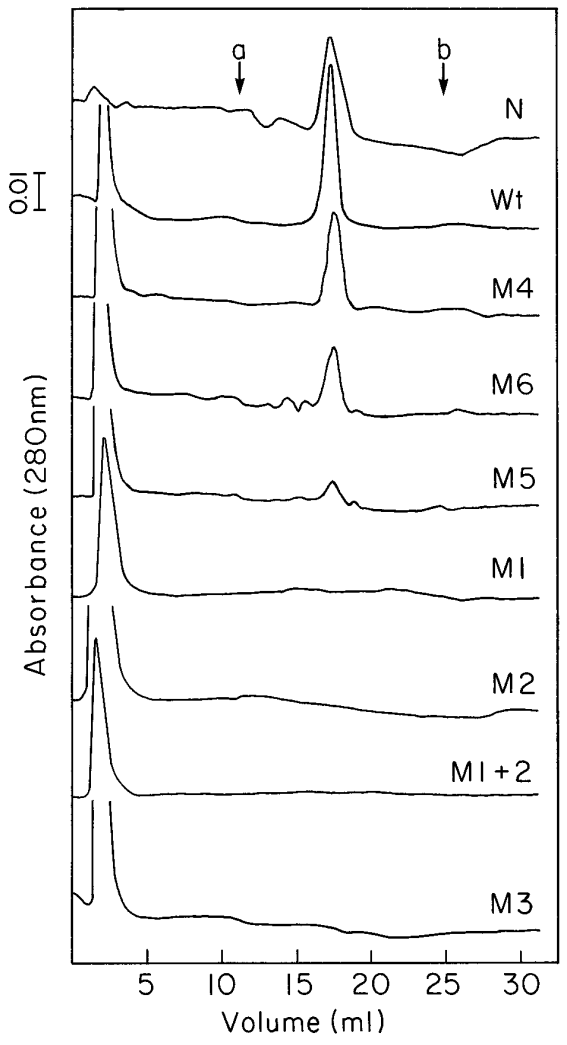

FIG. 5. **Heparin-Sepharose chromatography of native and recombinant FnNTDs.** Samples were loaded on prepacked columns and eluted with a 50–750 mM NaCl gradient as described under "Experimental Procedures." *Arrow a*, beginning of gradient; *arrow b*, end of gradient. Samples and total amounts loaded were: native FnNTD (*N*; 0.025 mg); Wt, wild type recombinant FnNTD (0.039 mg); M1 FnNTD (0.024 mg); M2 FnNTD (0.045 mg); M1+2 FnNTD (0.022 mg); M3–M6 FnNTDs, (0.032 mg).

conformationally abnormal molecules in these recombinant protein preparations (see "Discussion").

In contrast, mutations of basic residues in the Ω-loops (with a single exception) entirely abrogated normal binding of the FnNTD to heparin. The proteins encoded by clones M1 and M1+2 did not bind to heparin at all, whereas those encoded by M2 and M3 contained a subcomponent that eluted in a broad peak at salt concentrations lower than the normal value (Fig. 5). In the one exception, approximately 10% of the protein encoded by M5 eluted at the normal position, suggesting that like the targeted arginines outside the Ω-loops, the arginine in the fourth Ω-loop (the site of M5) is not a major determinant of heparin binding of the FnNTD. These results confirmed our interpretation of earlier chemical modification studies in which blocking the four most exposed arginines in the FnNTD had little effect on heparin binding by the fragment, but as less exposed arginines were successively modified, binding activity was rapidly lost (20).

Matrix-Driven Translocation Promoting Activity of the FnNTDs—Several of the recombinant FnNTDs were compared with the native fragment for their ability to interact with heparin or heparin-mimetic polystyrene on the surfaces of beads so as to induce MDT (24) (Fig. 6). We were particularly interested in whether the removal of basic residues from the Ω-loops destroyed the ability of the fragment to promote this effect, as previously inferred from chemical modification studies (20). Whereas the wild type recombinant fragment was fully active in promoting MDT, neither M1 nor M1+2 had MDT promoting activity. Unexpectedly, given its poor heparin binding activity (Fig. 5), M2 was able to promote MDT.

Conformational Properties of the Ω-Loop Mutants—The CD spectra of several of the Ω-loop mutants, in the absence and the presence of heparin, were compared with those of the Wt protein to gain some insight into the basis for the differences in their capacity to promote MDT. As noted above, the far UV spectrum of the Wt fragment exhibited normal mean residue ellipticity at 228 nm. Its minimum value was also close to normal, attaining a value of -15×10^2 deg cm^2 dmol^{-1} at 210 nm (Fig. 7), as compared with the value of -5×10^2 deg cm^2 dmol^{-1} for the native fragment at 212 nm (43–45, 49). For these measurements, in contrast to the thermal denaturation studies described above, heparin nonbinding components of the Wt preparation were not removed. The essential normality of the Wt ellipticity profile in Fig. 7 suggests that the conformationally abnormal protein molecules that are presumed to constitute nonbinding component in this preparation (Fig. 5) were not grossly misfolded.

The positive and negative ellipticity bands of the Wt spectrum were used as landmarks in comparing the mutant FnNTDs. Each of the Ω-loop mutants analyzed exhibited ellipticity profiles that were qualitatively similar to those of the native and wild type FnNTDs but had positive or negative peak ellipticity values that diverged from the normal values. Numerical estimation of the secondary structures of the various

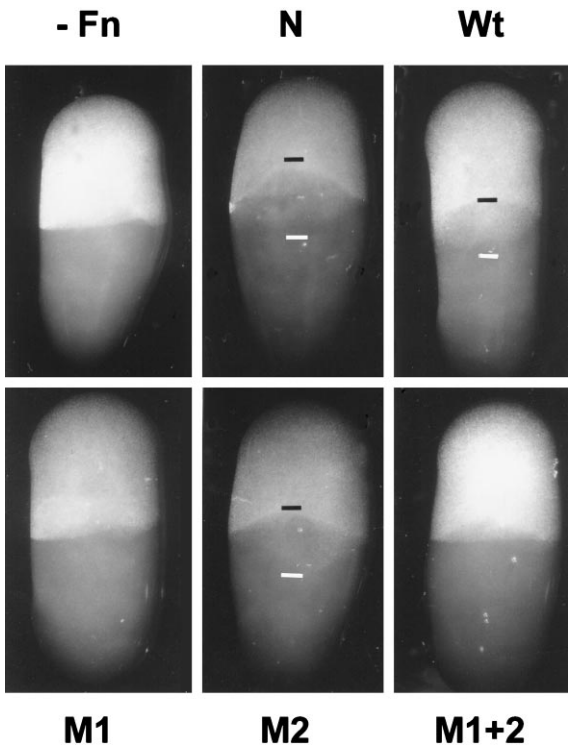

FIG. 6. **Matrix-driven translocation assays of recombinant FnNTDs.** All primary gels contained 5×10^6 polystyrene latex beads/ml. Secondary gels contained no fibronectin (*−Fn*); or 6 μg/ml of the following proteins: native FnNTD (*N*), Wt FnNTD, M1 FnNTD, M2 FnNTD, and M1+2 FnNTD. Movement of the interface, where it occurred (*N*, *Wt*, and *M2*), is indicated by *brackets*. These results were obtained in three or more trials with two independent preparations of recombinant FnNTDs.

FnNTDs by the CDESTIMA program suggested that M1 and M2 were only moderately altered with respect to content of β-sheet, the most prominent structural motif in the FnNTD (45, 50). The proportion of β-sheet in M2 was within 6%, and in M1 within 13%, of the wild type value. In contrast, M1+2 differed from Wt in the percentage of β-sheet by 43% (Table I).

We also determined the ellipticity profiles of the wild type and mutant FnNTDs in the presence of heparin, because we had previously found that the spectrum of the native fragment exhibited alterations in the presence of the glycosaminoglycan that were distinct from those induced by the artificial sulfated polysaccharide dextran sulfate and that, like the capacity of the FnNTD to interact with heparin-coated beads and induce MDT, were suppressible by Ca^{+2} (44). We found that the Wt spectrum exhibited the same characteristic changes in the presence of heparin as previously reported for the native FnNTD (44): a reduction in the amplitude and small red shift of the positive peak and an accentuation of the negative peak (Fig. 7). The ellipticity profile of protein M1 underwent similar qualitative changes (Fig. 7), but its content of β-turn increased in the presence of heparin, a result not seen with the Wt protein (Table I). The spectrum of M2 exhibited the heparin-induced red shift but no alterations in the amplitudes of its positive and negative bands, which were within the normal "heparin-induced" range whether or not heparin was present (Fig. 7). Protein M1+2 exhibited little change in the presence of heparin, the amplitudes of its negative band and its percentage of β-sheet and β-turn remaining well outside the normal range (Fig. 7 and Table I).

DISCUSSION

We have shown that removal of single positively charged amino acid side chains from certain Ω-loops of the type I modules of the FnNTD alters the capacity of the domain to interact with heparin, whereas removal of pairs of arginine side chains at either of the most prominent candidate sites for heparin binding outside the small loops has little effect on this interaction. The centrality of the Ω-loops in the heparin binding activity of the FnNTD was previously predicted on the basis of chemical modification studies of this fibronectin domain, in which it was found that blocking of the four most highly exposed arginines in the domain left both heparin binding and MDT promoting activity relatively intact, but modification of one or more additional arginines (presumed, on the basis of computed hydrophilicity, to be in the minor loops) dramatically reduced both activities (20).

In judging whether any given mutation exerted its effect on heparin binding of the FnNTD by virtue of its alteration of a specific ligand binding site or its induction of an abnormal conformation in the protein, we have made use of several considerations, both direct and indirect. In the first place, all of the substitutions we have made were for arginines or a lysine, residues that are not expected to be major determinants of the protein's folded state. In the case of mutant proteins M4 and M6, where normal heparin binding affinity was exhibited by a substantial proportion of the molecules (Fig. 5), it can reasonably be assumed that the mutations are consistent with relatively normal conformation of the FnNTD, although this conformation may not have been as readily attained or retained as in the Wt recombinant molecule.

For the proteins mutated in the Ω-loops of the type I modules other considerations hold. In the first place, such loops are considered to be independently folding surface features of the proteins in which they occur (27). Indeed, a survey of NMR-determined structures of 10 naturally occurring type I modules indicates that the overall structure of the module is unaffected by the sequence diversity and conformational flexibility of the Ω-loops (25). Thus if a conformational change were induced by the amino acid substitutions in proteins M1, M2, M3, or M5, they would be expected to be confined to an individual Ω-loop or perhaps affect interactions among such loops (20). In any case, the presence of such alterations would not undermine the inference of this study that the Ω-loops of the type I modules and their intrinsic or heparin-induced interactions are the major determinants of heparin binding in the FnNTD. Secondly, we note that the thermal denaturation profile of protein M1 is relatively normal (Fig. 4), as are features of the ellipticity profiles of this protein and M2 (Fig. 7 and Table I), confirming that alteration of individual basic Ω-loop residues in the FnNTD does not lead to gross misfolding of the protein.

In addition to the five type I modules of the FnNTD, only nine other such modules have been identified in all proteins surveyed to date: seven additional ones in fibronectin, one in factor XII (25), and one in tissue plasminogen activator (51). The minor loops of the type I modules, 10 or 11 residues in length and defined by flanking disulfide-bonded cystine pairs, are classic Ω-loops (27) and as such are likely to play a role in protein-ligand interactions. Although it has been noted that the exposed hydrophilic residues tend to vary among the different fingers (25), human fibronectin contains the sequence Gly-Arg-Gly in five of the Ω-loops of its 12 type I modules (two in the FnNTD), the sequence Gly-Lys-Gly in two others (one in the FnNTD), and sequences such as Gly-Ser-Arg-Gly in other corresponding positions, suggesting a functional importance in these Ω-loops for positively charged residues in a flexible environment. Of the four minor loops in the FnNTD that we modified, loss of arginine side chains in Ω-loops 1 or 2 (M3 and M1) essentially eliminated heparin binding, as did substitution of the lysine in Ω-loop 3 for a glutamic acid (M2). In contrast, the

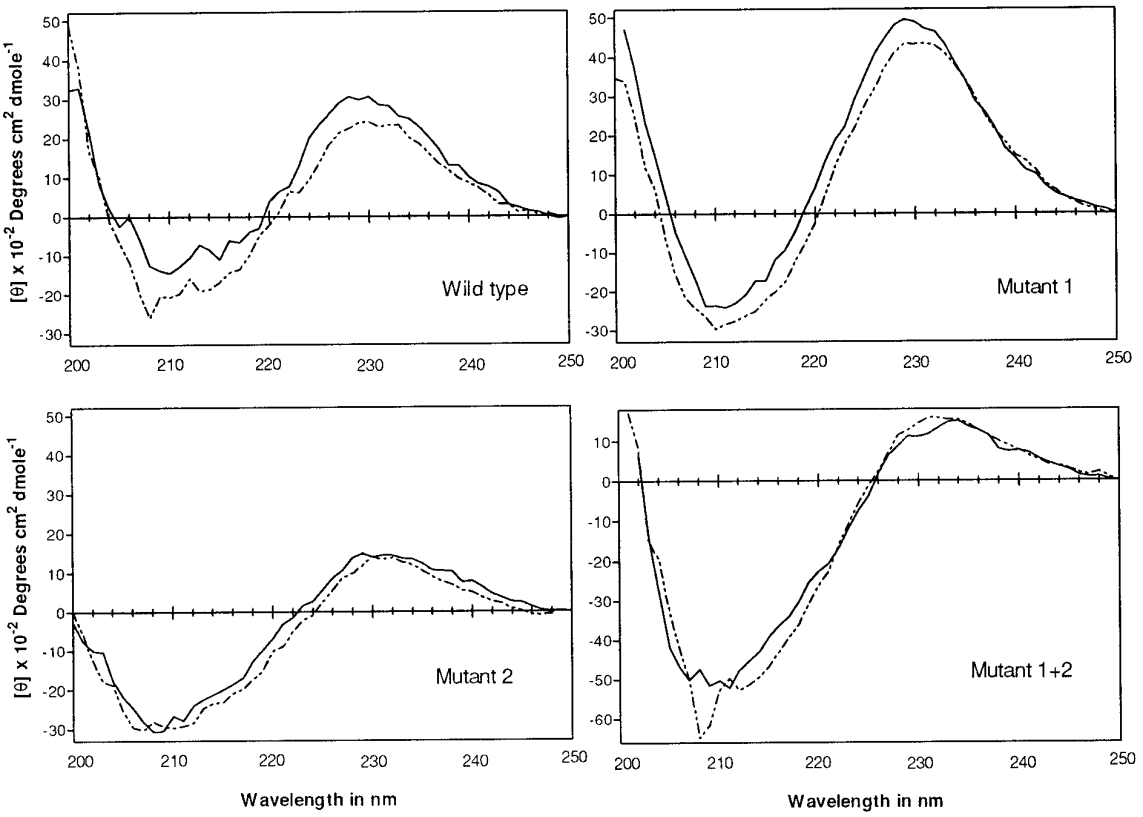

FIG. 7. **Far UV circular dichroic spectra of recombinant FnNTDs in the absence and the presence of heparin.** Spectra are shown of wild type (*upper left*), M1 (*upper right*), M2 (*lower left*), and M1+2 (*lower right*) proteins. *Solid* and *dashed lines* represent spectra of proteins obtained in the absence and the presence of heparin, respectively. In the runs shown, the heparin to protein weight ratio was 0.025. See "Experimental Procedures" for additional details concerning sample preparation.

TABLE I

Calculated percentages of secondary structural forms in wild type and mutant FnNTDs in the presence and the absence of heparin

Estimations from CD spectra according to the method of Yang *et al.* (38); see "Experimental Procedures." H, heparin.

	α-Helix	β-Sheet	β-Turn	Aperiodic
			%	
Wt	0	42.0	33.0	25.0
Wt + H	0	40.0	31.5	28.5
M1	0	47.5	33.0	19.5
M1 + H	0	37.5	38.0	24.5
M2	0	44.5	26.5	29.0
M2 + H	0	42.5	26.5	31.0
M1+2	0	60.0	13.0	27.0
M1+2 + H	0	54.0	17.5	28.5

small amount of M5-encoded protein that bound to the heparin column eluted at the normal position in the salt gradient (Fig. 5), suggesting that the properly folded molecules in this population retained full heparin binding capability. This indicates that the arginine side chain of Ω-loop 4 is not as importantly involved in heparin binding as the Ω-loop basic residues in modules I-1, I-2, and I-3 and may reflect its greater proximity to the cystine pair defining the loop base, compared with the basic residues in other Ω-loops, and its presence in a less flexible environment (one of its flanking residues is isoleucine, instead of the glycines or serines flanking the basic residues in the other FnNTD Ω-loops). At this time we have no evidence concerning the role of the arginine residue in I-5, but because it appears in a repeat of the flexible Gly-Arg-Gly motif, it would be expected to contribute to the heparin binding activity of the FnNTD.

Although the binding of the domain to heparin is relatively weak (52), heparin-coated particles placed in tissue culture accumulate at fibronectin-rich foci in a fashion that depends on interaction of the bead surface with the FnNTD (21). Moreover, the FnNTD interacts with heparin on particle surfaces to induce MDT only under conditions of low divalent cation concentration permissive for a heparin-induced conformational change in the fragment (44). The conformationally altered protein is presumed to promote bead redistribution in the cell cultures and model matrices by causing a reduction in free energy at the bead surface-matrix interface (21, 53–55).

We have previously hypothesized that the interaction of the FnNTD with heparin occurs through the cooperative interaction of two or more positively charged sites in the minor loops (20). The type I modules of the FnNTD are well known to undergo such conformational rearrangements; for example, fluorescence and CD studies have shown that the modules in recombinant pairs I-2-I-3 and I-4-I-5 of the domain undergo structural changes as a result of interactions with fibrin, which are required for or enhance binding to that ligand (9, 10). Although structural studies of the recombinant fibronectin type I-4–5 pair indicate that these two modules, in isolation, combine with a fixed and intimate hydrophobic contact (50, 56), the relationship among the various modules when the intact FnNTD is bound to heparin is likely to be different. Our earlier CD and fluorescence anisotropy studies indicated that under conditions of low divalent cation concentration the interaction of the FnNTD with heparin causes measurable changes in the conformation and flexibility of the protein fragment (44, 49). Indeed, the ability of heparin to alter the structure of protein M1 under the stationary conditions of the CD measurements (Fig. 7 and Table I) despite the inability of this protein to bind heparin under flow conditions (Fig. 5) suggests that column binding may not be the the most favorable assay for productive interactions between fibronectin and heparin-like molecules at

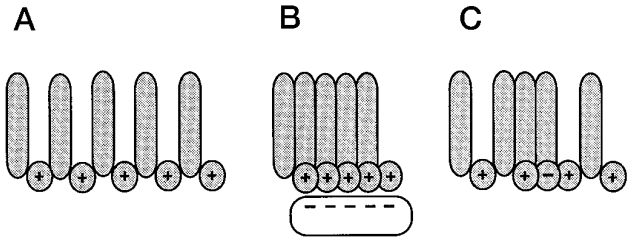

FIG. 8. **Model of loop-loop interactions in FnNTDs.** *A* represents FnNTD containing normal sequence in Ω-loops. In *B*, binding to heparin is proposed to induce more intimate interactions among the minor loops. In *C*, the Lys → Glu substitution in Ω-loop 3 of M2 is proposed to promote loop-loop interactions similar, in part, to those induced by heparin.

cell-matrix interfaces *in situ*. We propose that structural changes in the FnNTD induced by the Ω-loop mutations in the present study, along with the MDT assay, can provide insight into potentially important changes in fibronectin that can brought about by binding to heparin-like molecules.

The CD spectrum (Fig. 7) and predicted secondary structure (Table I) of M2, in which an Ω-loop lysine is replaced by a glutamic acid, were the most normal of all the mutants analyzed; this was an expected result, because the alteration consisted of the substitution of one charged residue for another. It was also not surprising that M2 was unable to bind to heparin (Fig. 5), because it contributes a negative charge in the putative binding pocket. What was unexpected, however, was the ability of this protein to promote MDT, which normally depends on the FnNTD-heparin interaction (20).

The unusual band of positive ellipticity at ~230 nm can provide comparative structural information for closely related proteins, such as wild type and mutated FnNTDs (48), because in addition to known contributions to this signal from aromatic chromophores (46, 48, 57, 58) and disulfide bonds (59, 60), it can also be amplified or attenuated by conformational changes (43, 48). Although the specific structural features that contribute to the amplitude of the ~230 nm band are not known, it is notable that it is reduced in magnitude in M2 relative to the value for the native and wild type proteins (Ref. 44 and Fig. 7) and becomes attenuated when native (44) or wild type (Fig. 7) FnNTD interact with heparin. Furthermore, interaction of M2 with heparin caused no additional attenuation of the band. We speculate that interactions between the mutated Ω-loop 3, which now contains a negatively charged residue, and unmutated Ω-loops containing positively charged residues, induce changes in the FnNTD that would normally occur when the domain binds to heparin (Fig. 8). This may help explain the similarity of the ellipticity profile of M2 to the heparin-induced profile of the native (44) and Wt (Fig. 7) fragments, and why, despite its failure to bind heparin (Fig. 5), this mutant protein is nonetheless capable of inducing MDT (Fig. 6). These considerations may also provide insight into morphogenetic interactions mediated by the FnNTD in living tissues (19, 61).

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