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Three-Dimensional Structural Analysis of Fibronectin Heparin-Binding Domain Mutations

Yvonne Kapila,[1,2]* Denise Doan,[1] Elizabeth Tafolla,[1] and Robert Fletterick[2]

[1]Department of Stomatology, University of California San Francisco, San Francisco, California 94143-0512
[2]Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, California 94143-0448

Abstract Using recombinant fibronectin proteins containing the V region and two point mutations in the high-affinity heparin-binding domain, we previously showed that these domains modulate tumor cell invasion as well as proteinase expression and apoptosis in human fibroblasts. Structurally, the wildtype counterparts to these two point mutations, together with four other discontinuous, positively charged residues, form a cationic cradle in domain III-13 of fibronectin that binds heparin. We constructed a three-dimensional model of this cationic cradle and determined whether the two engineered point mutations in the heparin-binding domain would alter this cradle conformation, thus explaining the altered cell behavior. Our model of fibronectin domain III-13 was generated from a template of the three-dimensional structure of a homologous (25% identity) domain, III-3, from tenascin. The amino acid sequences of III-13 that differed from tenascin III-3 were replaced, and side chains for positively charged arginines 6 and 7 were substituted with uncharged threonines. The model revealed that the two mutated threonine residues were solvent accessible, readily accommodated as part of an antiparallel beta strand, and remained part of the three-dimensional cradle. These models suggest that the two point mutations in the heparin-binding domain of fibronectin III-13 alter cell function probably through changes in charge and not through changes in the conformational structure of the cationic cradle. J. Cell. Biochem. Suppl. 36:156–161, 2001.

Key words: fibronectin; heparin-binding domain; point mutations; modeling

The extracellular matrix (ECM) molecule fibronectin (FN) is composed of several domains that mediate multiple cell functions through cell surface integrin and proteoglycan receptors. When isolated, specific domains of FN display activities not exhibited by the intact molecule. For example, the central cell-binding domain of FN (FN 120) induces rabbit synovial fibroblasts [Huhtala et al., 1995] and human fibroblasts [Kapila et al., 1996] to express elevated levels of matrix metalloproteinases (MMPs), whereas fragments from the amino-terminal and gelatin-binding domains induce chondrolysis in vitro, the latter effect presumably through MMP and serine proteinase induction [Homandberg et al., 1992; Xie et al., 1994]. These FN fragments are also associated with chronic inflammatory states in vivo, since high levels of such fragments have been found in synovial fluids from arthritic patients [Carsons et al., 1985; Griffiths et al., 1989; Xie et al., 1992] and in gingival crevicular fluid from patients with periodontitis [Talonpoika et al., 1989, 1993].

Another function recently attributed to FN is protection against programmed cell death, or apoptosis. We have recently identified some of the domains of FN that are critical to modulating this function [Kapila et al., 1999], as well as tumor cell migration and invasion [Kapila et al., 1997]. Using a series of recombinant FN proteins (Fig. 1A and B) which contain the V (variable) region and two point mutations in the high-affinity heparin-binding domain of FN, we found these domains to be critical to these two mechanisms.

In terms of the structural importance of the heparin-binding domain, it has been demonstrated that there is a three-dimensional catio-

*Correspondence to: Yvonne Kapila, D.D.S, Ph.D, Department of Stomatology, HSW 604, Box 0512, University of California San Francisco, San Francisco, CA 94143-0512. E-mail: ykapila@itsa.ucsf.edu

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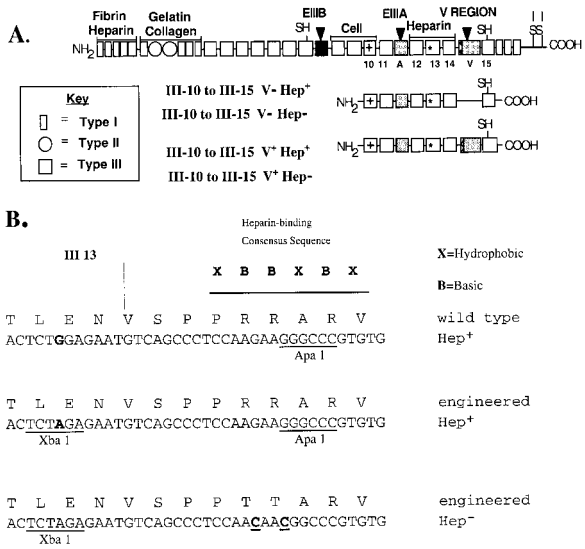

Fig. 1. Recombinant FN proteins. **A:** experiments were performed with four different recombinant FN protein fragments engineered to examine functions of the high-affinity heparin-binding domain and the alternatively spliced V (variable) region of FN. All four proteins span type III repeats 10 through 15. Two proteins contain the alternatively spliced V region (designated with a V^+ symbol), while the other two do not (designated with a V^- symbol). Two proteins also contain the high-affinity heparin-binding domain (designated with a H^+ symbol), while the other two proteins have a mutated, non-functional heparin-binding domain (designated with a H^- symbol). The RGD cell-binding sequence (+) and high-affinity heparin-binding sequence (*) are indicated within the clear boxes. The alternatively spliced EIIIA and V regions are shown as a gray shaded box and a rectangle, respectively. The key to the bottom left of the figure depicts the type of repeating structural domains within the FN molecule. **B:** the high-affinity heparin-binding domain was rendered non-functional by insertion of synthetic oligonucleotides between restriction sites for *Apa*1 and *Xba*1. These oligonucleotides preserved the native FN protein sequence but changed two adjacent arginines to threonines in the mutated protein. Positions of restriction sites are underlined. Altered nucleotides are indicated in bold type. The heparin-binding consensus sequence is indicated at the top of the figure. The sequences depict the wild-type FN sequence, the engineered Hep^+ sequence (nucleotide sequence contains a new restriction site for *Xba*1 but preserves the native FN protein sequence and heparin binding of the expressed protein) and the engineered Hep^- sequence (the two adjacent arginines are mutated to threonines, and heparin binding of the expressed protein is virtually lost).

nic cradle in the heparin-binding domain of repeat III-13 of FN [Busby et al., 1995]. We suspected that the two engineered point mutations in the heparin-binding domain of FN present in our recombinant proteins were altering this cradle structure and thereby altering cell behavior. To examine this possibility, we constructed a three-dimensional model of this cationic cradle and then made substitutions for the mutated residues to compare any changes in structure.

MATERIALS AND METHODS

Recombinant FN Proteins

Four recombinant FN proteins were tested in these experiments (Fig. 1). These proteins, described elsewhere [Kapila et al., 1997], either included (V+) or excluded (V−) the alternatively spliced V region and contained either an unmutated (Hep+) or a mutated, non-functional high-affinity heparin-binding domain (Hep−). All four proteins also contained the arginine, glycine, aspartic acid (RGD) cell-binding site and the alternatively spliced EIIIA domain of FN. These proteins were designated as V−H+, V−H−, V+H+, and V+H−.

Sequence

The sequence of FN type III-13 (Fig. 2) was obtained from rat fibronectin (SWISS-PROT=FINC-RAT). The sequence (Fig. 2) and three-dimensional model of tenascin III-3 (human tenascin) were retrieved from the protein data bank (1TEN).

Alignment

The alignment between the FN III-13 and tenascin III-3 sequence was done by using the alignment tool of the Expasy program. The sequences are 26.6% identical in a 79-residue overlap with a gap frequency of 1.3%. Hence, arginine was used to replace the amino acid residues at positions 6, 7, 9, 23, 47, and 54 while

FN Type III-13 Domain Sequence:
1NVSPPR$_6$R$_7$AR$_9$VTDATETTITISWR$_{23}$TK$_{25}$TETITCFQVDAIPANGQTPV
QR$_{47}$TISPDVR$_{54}$SYTITGLQPGTDKIHLYTLNDNARSSPVVIDAST 89

Tenascin Type III-3 Domain Sequence:
1RLDAPSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRT
TIDLTEDENQYSIGNLKPDTEYEVLISRRGDMSSNPAKETFTT 90

Sequence Alignment between FN III-13 and Tenascin III-3 Domain:
5 PRRARVTDTETITITSWRTKTETITGFQVD-AIPANGQTPVQRTISPDVRSYTITGLQP 64
5 PSQIEVKDVTDTTALITWFKPLAEIDGIELYGIKDVPGDRTTIDLTEDENQYSIGNLKP 64

Heparin-Binding Cradle on FN III-13:
ARG$_6$(R$_6$), ARG(R$_7$), ARG$_9$(R$_9$), ARG(R$_{23}$), LYS$_{25}$(K$_{25}$), ARG$_{47}$(R$_{47}$)AND ARG$_{54}$(R$_{54}$)

Fig. 2. Sequence alignment. The sequence of FN type III-13 was obtained from the rat fibronectin (SWISS-PROT = FINC-RAT). Residues that were to be mutated or substituted are italicized and denoted by numerical subscripts. The sequence of tenascin III-3 (human tenascin) was retrieved from the protein data bank (1TEN). Sequence alignment between FN III-13 and tenascin III-3 domains is illustrated. Mutations and substitutions in the cationic cradle of the heparin-binding domain of FN are illustrated.

lysine was used to replace the residue at position 25 on the tenascin III-3 domain sequence. Then the three-dimensional structure of the FN III-13 domain was modeled after the three-dimensional structure of the tenascin III-3 domain.

Three-Dimensional Model

Using the Insight II 97.0 software, the Arg6 and Arg7 in the heparin-binding cradle on the three-dimensional model of FN III-13 were replaced by threonine residues in their canonical rotamers. The three-dimensional model of the mutated heparin-binding cradle of FN III-13 was then generated with the threonine sidechains positioned in stable conformers that reached into the solvent. Subsequently, the three-dimensional models of the Hep+ and Hep− heparin-binding cationic cradle of FN were rotated around the X, Y, and Z-axis and compared from different angles to assess the effect of the mutations on the heparin-binding cradle. No significant short contacts were present in the model, since we chose to maintain most of the tenascin III structure.

Electrostatic Potential

The program Grasp was used to measure the differences in the electrostatic potential between the wildtype and mutated heparin-binding cationic cradle of FN. The wildtype and mutated FN III-13 regions were entered into the program by converting the Insight files to PDB files. Grasp was then used to compute and analyze the difference in the electrostatic potential at the protein surface of these two structures using the Poison Boltzman formulation in Delphi. This method does not account for the changes in electrostatic potential at the cradle that might occur from differences between the modeled and the native FN structure.

RESULTS

The three-dimensional models of the unmutated (Hep+) and mutated (Hep−) heparin-binding cationic cradle of FN were rotated around the X, Y, and Z-axis and compared from different angles to assess the effect of the mutations on the heparin-binding cradle (Figs. 3–5). The models revealed that the two mutated threonine residues were solvent accessible, readily accommodated as part of an antiparallel beta strand, and remained part of the three-dimensional cradle. In our model, the two point mutations made in the cationic cradle of the heparin-binding domain of the recombinant FN proteins did not significantly alter the three-dimensional structure of this cradle.

Lastly, the electrostatic surface potential of both the Hep− and Hep+ forms of the cationic cradle were compared, and again the overall charge on the protein surface did not change significantly with the introduction of the two point mutations (data not shown). This result is expected since the arginines and replacement threonine residues are solvent accessible in the two models. The solvent, modeled as 0.15 M salt, screens the effects of the two plus charges that are absent in the mutated form.

DISCUSSION

We initially suspected that the significant effect on both cell survival and migration by the mutated heparin-binding domain-containing FN fragment was due to its potential alteration of the three-dimensional structure of the cationic cradle within the heparin-binding domain. However, analysis of these models visually and by simple nearest neighbor bump checks suggested that the two point mutations in the heparin-binding domain of fibronectin III-13 did not significantly alter the cradle structure. Therefore, the mutant FN protein (Hep−) was altering cell function without apparently altering the conformational structure of the cradle. This suggested that there were other important interactions taking place between the recombinant FN protein (V+H−) and the cell surface that were accounting for the altered cell function.

Therefore, to test whether other surface interactions between the FN protein V+H− and the cells might be contributing to the observed altered cell behavior, electrostatic surface potentials were calculated and compared for the mutant and wildtype cradle structures. However, this analysis also revealed little difference in the electrostatic potentials between the wildtype and the mutant models (data not shown). There are several reasons why the charge effects and overall electrostatic surface potentials may appear minimal. One reason is that the cluster of plus charges that contain the substitutions on the mutant structure are likely solvated and water dissipates the charge. Surface charge changes are less sig-

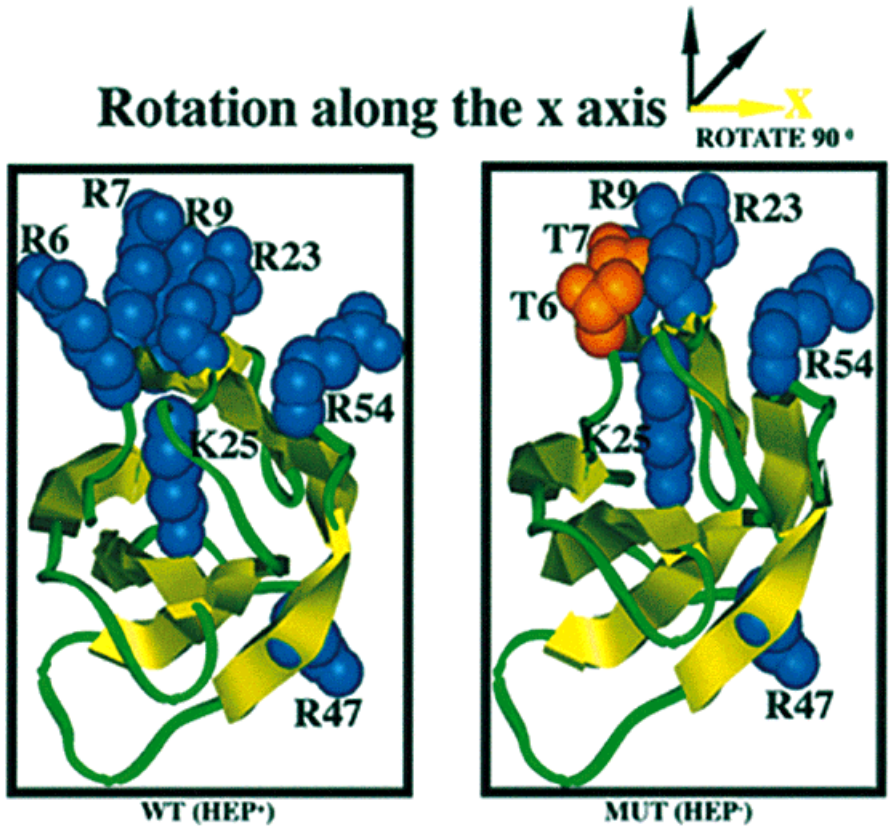

Fig. 3. Three-dimensional model: 90° rotation along the x-axis. Blue spheres represent substituted side chains and red spheres represent mutated side chains. Thin ribbons represent α helices and the broad ribbons represent β strands. The wildtype (WT) structure is illustrated on the left and the mutant (MUT) structure is illustrated on the right.

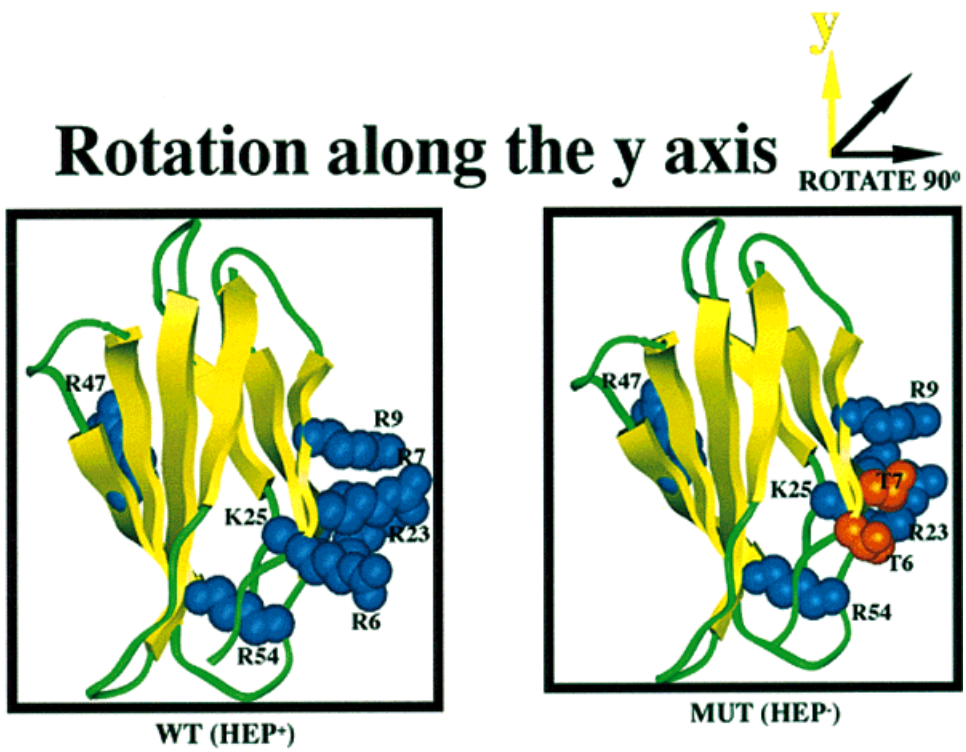

Fig. 4. Three-dimensional model: 90° rotation along the y-axis. See Figure 3 for explanation of structures.

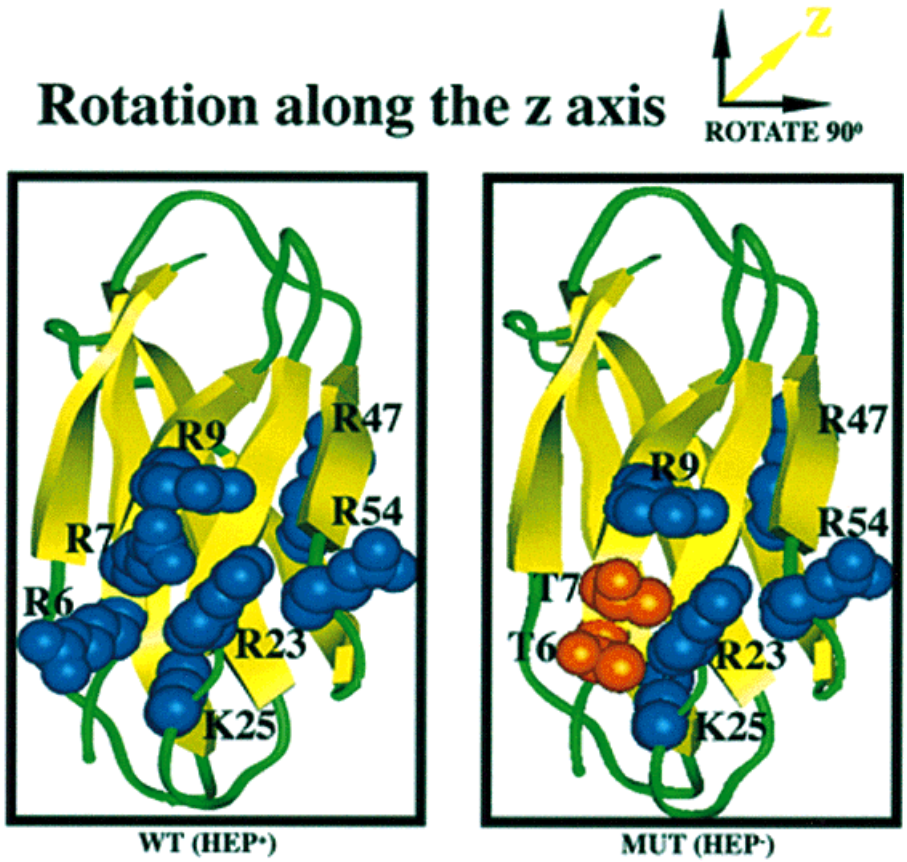

Fig. 5. Three-dimensional model: 90° rotation along the z-axis. See Figure 3 for explanation of structures.

nificant than changes nearly embedded in the surface. The dielectric near or under the surface is 4.0 and outside is 80.0 — a factor of 20 in the force. A second reason is that the differences may not be thermodynamic but kinetic. The kinetics of association could change for the mutant structure, since as two molecules approach each other the dielectric at the interface changes as desolvation progresses. Lastly, these calculations are based on modeling derived from sequence homology, which may not accurately predict sidechain conformations for region III-13 of FN or the effects of a possibly different shape in wildtype FN, and therefore strongly affect the electrostatic calculations.

In summary, these models suggest that the two point mutations in the heparin-binding domain of fibronectin III-13 alter cell function probably through charge alterations and not through changes in the conformational structure of the cationic cradle.

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REFERENCES

Busby TF, Argraves WS, Brew SA, Pechik I, Gilliland GL, Ingham KC. 1995. Heparin binding by fibronectin module III-13 involves six discontinuous basic residues brought together to form a cationic cradle. J Biol Chem 270:18558–18562.

Carsons S, Lavietes BB, Diamond HS, Kinney SG. 1985. The immunoreactivity, ligand, and cell binding characteristics of rheumatoid synovial fluid fibronectin. Arthritis Rheum 28:601–612.

Griffiths AM, Herbert KE, Perett D, Scott DL. 1989. Fragmented fibronectin and other synovial fluid proteins in chronic arthritis: their relation to immune complexes. Clin Chim Acta 184:133–146.

Homandberg GA, Meyers R, Xie D-L. 1992. Fibronectin fragments cause chrondrolysis of bovine arti-

cular cartilage slices in culture. J Biol Chem 267:3597–3604.

Huhtala P, Humphries MJ, McCarthy JB, Tremble PM, Werb Z, Damsky CH. 1995. Cooperative signaling by α5β1 and α4β1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. J Cell Biol 129:867–879.

Kapila YL, Kapila S, Johnson PW. 1996. Fibronectin and fibronectin fragments modulate the expression of proteinases and proteinase inhibitors in human periodontal ligament cells. Matrix Biol 15:251–261.

Kapila YL, Niu J, Johnson PW. 1997. The high-affinity heparin-binding domain and the V region of fibronectin mediate invasion of human oral squamous cell carcinoma cells in vitro. J Biol Chem 272:18932–18938.

Kapila YL, Wang S, Johnson PW. 1999. Mutations in the heparin-binding domain of fibronectin in cooperation with the V region induce decreases in $pp125^{FAK}$ levels plus proteoglycan-mediated apoptosis via caspases. J Biol Chem 274:30906–30913.

Talonpoika J, Heino J, Larjava H, Hakkinen L, Paunio K. 1989. Gingival crevicular fluid fibronectin degradation in periodontal health and disease. Scand J Dent Res 97: 415–421.

Talonpoika J, Paunio K, Soderling E. 1993. Molecular forms and concentration of fibronectin and fibrin in human gingival crevicular fluid before and after periodontal treatment. Scand J Dent Res 101:375–381.

Xie D-L, Hui F, Meyers R, Homandberg GA. 1994. Cartilage chondrolysis by fibronectin fragments is associated with release of several proteinases: stromelysin plays a major role in chondrolysis. Arch Biochem Biophys 311:205–212.

Xie D-L, Meyers R, Homandberg GA. 1992. Fibronectin fragments in osteoarthritic synovial fluid. J. Rheumatol 19:1448–1452.