Fibronectin Conformation and Assembly: Analysis of Fibronectin Deletion Mutants and Fibronectin Glomerulopathy (GFND) Mutants

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ABSTRACT: To study fibronectin (FN) conformation and assembly, we generated several deletion mutants: FNΔ1−5, FNΔIII1−3, FNΔIII4−8, and FNΔIII1−14. A monomeric form, FNmono, which lacked the C-terminal dimerization region, was also created. FNtnA−D was generated by swapping FNIII domains 1−8 in FNΔIII11−14 with seven FNIII domains from tenascin-C. The conformations of these mutants were analyzed by glycerol gradient sedimentation under low-salt (20 mM NaCl) and high-salt (200 mM NaCl) conditions. Surprisingly, most of the mutants showed a compact conformation under low-salt conditions, except for FNtnA−D. When we tested these mutants in cell culture, FNΔ1−5, FNΔIII1−3, and FNtnA−D were unable to form a matrix. Interestingly, FNΔIII1−3 and FNtnA−D were capable of co-assembly with full-length FN, while FNΔ1−5 was not. This indicates that the segment I1−5 is crucial for matrix assembly and segment III1−3 is also important. Mutations in FN are associated with glomerulopathy, but when we studied mutant proteins, the single-nucleotide mutations had only minor effects on conformation and matrix assembly. The mutations may destabilize their FNIII domains or generate dimers of dimers by disulfide cross-linking.

Plasma fibronectin (FN) is secreted by hepatocytes in the liver and circulates in blood. Plasma FN is a dimer, each subunit comprising 12 FN type I domains, two FN type II domains, 15 FN type III domains, and a variable domain (V), although one of the subunits in the dimer lacks V because of alternative splicing. The conformation of plasma FN has been extensively studied over the past several decades. It forms a compact conformation under physiological conditions, but at high ionic strength or high pH, the conformation changes to an extended form. The exact structure of this compact conformation is not known and may represent multiple states; in this paper, we use the term “compact conformation” to refer to the state (or states) of the protein that sediments faster at a low ionic strength. It has been proposed that an interaction between III2−3 and III12−14 induces the compact conformation. This interaction was originally discovered by the dimerization of FN fragment III12−14. It is important to note that this dimerization does not occur at a physiological salt concentration (150 mM NaCl), which suggests that the compact conformation is stabilized by interactions in addition to III2−3 and III12−14. An earlier study indicated that I1−5 interacted with III12−14 to form the compact conformation. An interaction between I1−5 and III3 was also suggested, although this interaction was extremely weak at a physiological salt concentration. Maurer et al. recently showed that a I1−5 binding peptide, which was engineered from a bacterial adhesin, induced the extended FN conformation in solution. This also suggests that I1−5 is involved in forming the compact conformation.

To study the function of plasma FN, tissue specific knockout mice were created to deplete FN in blood. Surprisingly, these mice did not show any obvious abnormality and had normal skin wound healing. However, transient focal cerebral ischemia was more severe in the knockout mice, indicating that plasma FN plays a role in brain wound healing. Plasma FN is also important for thrombus initiation, growth, and stability in injured arterioles.

Soluble FN assembles into insoluble fibrils, called an FN matrix, on the cell surface. Detergent-insoluble FN matrix fibrils appear to be formed by noncovalent bonds. However, the exact locations of potential bonds and the interfaces between molecules are still not known. Because soluble FN, such as plasma FN, cannot assemble without cells, it is thought that one or more cryptic assembly sites are exposed only when FN binds to the cell surface. Electrostatic bonds between dimers might account for noncovalently bonded multimers, although these bonds may not be strong enough to hold the molecules together during detergent extraction. We have previously proposed that FN aggregation and assembly require the unfolding of FNIII domains. In this model, high local FN concentrations on the cell surface could enhance the probability of interactions following the spontaneous unfolding and/or opening of FNIII domains.

A recent genetic study showed that a hereditary kidney disease, FN glomerulopathy (or glomerulopathy with FN deposits [GFND]), was caused by mutations in the FN1 gene. Single-nucleotide polymorphisms were found in domains III4 and III13.
Because nonfibrillar FN deposits in GFND patients were mainly derived from plasma FN, the authors suggested that these mutations may affect the conformation of plasma FN, which more easily formed abnormal nonfibrillar FN deposits in the glomerular matrix. More recently, additional mutations were found in III4 and III13, and one mutation was found in III9.18

In the study presented here, we generated several FN deletion mutants and GFND mutants, to study the domains responsible for forming the compact FN conformation and assembling the matrix.

**MATERIALS AND METHODS**

**Mutant Construction and Mammalian Cell Protein Expression.** For full-length FN expression, we used the FN-YFP vector, FN-YPet/pHLSec2 in which mYPet (a YFP variant) was inserted between III6 and III7, for visualization and quantification.16 This vector has an engineered signal sequence that gives an additional three amino acids (EGS) at the N terminus of the secreted protein. To generate the various deletion mutants, shortened FN fragments were amplified by polymerase chain reaction and cloned back into the FN-YPet/pHLSec2 vector: FNΔI1−5 (deletion; Q32AQQ···KCER272), FNΔIII1−3 (deletion; V614FIT···TTGT901), FNΔIII4−8 (deletion; P902RSD···RQKT1447), FNΔIII11−14 (deletion; P1636SQM···RKKT2082), and FNmono (deletion; C2458PIE···DSRE2477). Diagrams of these mutants are shown in Figure 1. The additional restriction enzyme sites for AscI and NotI were created for cloning. For amino acid numbering, we used RefSeq NP_997647, which corresponds to that used in previous studies.17 We also created an FN-tenascin-C chimeric construct, FNtnA−D, in which FNIII domains 1−8 in FNΔIII11−14 were replaced with FNIII domains A−D from tenascin-C (the seven main alternatively spliced FNIII domains, E1072QAP···IATT1708; CAA55309 was used for the numbering). A previous study showed that domains A−D do not interact with FN.19 For co-assembly experiments, an FN-CFP construct was created by replacing mYPet in the FN-YFP construct with mECFP.20 We also generated the GFND mutants (Y973C, W1925R, and L1974R) by mutagenesis using the wild-type FN (without YFP) expression construct.21 These constructs were expressed in a transient mammalian cell expression system as reported previously.16,21,22 Briefly, purified constructs were transfected into HEK293 cells with PEI (polyethylenimine-25 kDa branched, Aldrich). The conditioned medium was collected after transfection for 6−7 days. FN, FN-YFP, and the mutants were purified with a gelatin-agarose column using 4 M urea for elution. To exchange the buffer, purified proteins were dialyzed against PBS {phosphate-buffered saline [2.7 mM KCl, 1.47 mM KH2PO4, 8.1 mM Na2HPO4, and 137 mM NaCl (pH 7.4)]}. The yields of proteins varied from ∼0.5 to ∼1.5 mg from ∼100 mL of culture medium, obtained from three 150 cm2 tissue culture flasks. The insertion of YFP reduced protein expression levels, and some deletions or mutations decreased expression levels even further.

**Glycerol Gradient Sedimentation.** The sedimentation coefficients of the FN mutants were estimated by velocity sedimentation through 5 mL 15 to 40% glycerol gradients as reported previously. Gradients contained 1 mM PIPES (pH 7.0) and either 20 mM NaCl (low salt) or 200 mM NaCl (high salt); 150 μL samples were applied to the gradients and centrifuged at 38000 rpm for 16 h in a Beckman SW-55 Ti rotor at 20 °C. For calibration, catalase (11.3 S), aldolase (7.3 S), bovine serum albumin (4.6 S), and ovalbumin (3.5 S) were added to the samples as internal standards. Fractions were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (10% acrylamide) under nonreducing conditions (2X loading buffer containing 10 mM iodoacetamide). These experiments were repeated three times under both low- and high-salt conditions. The peak fractions of samples and standards were determined by the intensity of Coomassie blue.

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**Figure 1.** Diagrams of FN-YFP and the deletion mutants used in this study. Predicted unstructured regions are shown as lines. The locations of engineered restriction enzyme sites for cloning are shown with arrows.

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stained bands. For example, under low-salt conditions, FNΔIII4–8 was in fraction 4.0, catalase was in fraction 3.9, aldolase was in fraction 6.4, BSA was in fraction 9.2, and ovalbumin was in fraction 9.9 (Figure 3). On the basis of these results, a standard curve ($S = -1.2532 \times \text{fraction} + 15.886$) was generated and the $S$ value of FNΔIII4–8 was calculated to be 10.9.

**Cell Culture and FN-YFP Quantification Using YFP Fluorescence.** FN(–/–) cells were maintained with DMEM (Sigma) containing 10% fetal calf serum (FCS, Sigma). Cells were harvested with 0.05% trypsin and 2 mM EDTA (Invitrogen) and rinsed with DMEM containing 2 mM PMSF to quench the trypsin activity. Cells were then resuspended with DMEM containing 1% FCS that was depleted of FN by being passed through a gelatin column. Cell suspensions (0.4 mL, $5 \times 10^5$ cells/mL) containing 30 nM FN-YFP or the deletion mutants were plated in a 24-well culture plate. For microscopy, 12 mm circular cover glasses were added to several wells, mutants were plated in a 24-well culture plate. For microscopy, 12 mm circular cover glasses were added to several wells, fixed, and mounted on slides after being cultured for ~16 h.

The concentration of proteins was determined from the YFP absorbance at 514 nm with the molar extinction coefficient for mYPet of $85000 \text{M}^{-1} \text{cm}^{-1}$, 20. The concentrations of FN-YFP and mutants are given for the monomer. To assay for incorporation of the YFP-labeled protein into the matrix, cells were cultured for ~16 h, and then each well was rinsed three times with 0.5 mL of PBS containing Ca$^{2+}$ and Mg$^{2+}$. Cells were then treated with 0.1 mL of trypsin (10 μg/mL) in PBS containing 5 mM EDTA for 30 min at room temperature. Trypsin digestion was quenched with 2 mM PMSF, and samples were centrifuged at 15000 rpm for 10 min to remove the cells. The total culture YFP was then estimated from the fluorescence of the supernatant. For background subtraction, the same experiments were performed without the cells. The fluorescence was assayed with a Shimadzu RF-5301-PC spectrofluorometer. Samples were excited at 514 nm, and emission was recorded at 528 nm with slit widths of 3 nm for excitation and 5 nm for emission. These measurements were performed at room temperature with quadruplicate samples in each plate. For co-assembly experiments, 15 nM FN-CFP was mixed with 15 nM YFP-labeled mutant FN. The concentration of FN-CFP was determined from the absorbance at 433 nm with a molar extinction coefficient for monomeric ECFP* of 23500 $\text{M}^{-1} \text{cm}^{-1}$. After solubilization of the fluorescent proteins, the YFP fluorescence was measured as described above, while the CFP fluorescence was measured by exciting samples at 433 nm and recording emission at 475 nm with slit widths of 5 nm for excitation and 10 nm for emission. For microscopy, some samples were fixed with 3.7% formaldehyde in PBS, rinsed with PBS, and mounted on slides. Incorporation of 30 nM recombinant FN or the GFND mutants was detected by immunostaining. After fixation, samples were stained with an anti-FN polyclonal antiserum (HBS, 1:1000) and an Alexa Fluor 488 anti-rabbit IgG antibody (1:1000). The samples were observed with a light microscope (Zeiss Axioplan, objective EC Plan-Neofluar 40×/1.3 oil), and the images were captured with a cooled CCD camera (CoolSNAP HQ, Roper Scientific) using AxioVision software (Zeiss) and processed with Photoshop (Adobe).

**RESULTS**

**Conformations of the FN Deletion Mutants.** The YFP-tagged FN and the deletion mutants used in this study are depicted in Figure 1. The quality of the purified FN-YFP and deletion mutants was analyzed by SDS–PAGE (5% acrylamide) under nonreducing and reducing conditions (Figure 2). The majority of the proteins were verified to be disulfide-bonded dimers with minor proteolysis fragments. The sedimentation coefficients for the FN mutants were determined in 1 mM PIPES (pH 7.0) with 15 to 40% glycerol gradients containing either 20 mM NaCl (low salt) or 200 mM NaCl (high salt) as reported previously.26 An earlier study by Rocco et al.26 showed that in 30% glycerol (the midpoint of our gradient) with 20 mM NaCl, FN had a compact conformation similar to that at a physiological salt concentration (150 mM NaCl) with no glycerol. Similarly, in 200 mM NaCl with glycerol, FN has an extended conformation equivalent to >500 mM NaCl alone. After sedimentation, the collected fractions were analyzed by SDS–PAGE (10% acrylamide) and the sedimentation coefficients were determined by internal standards. We often saw some proteolysis fragments in this assay (the C-terminal portion of FN is particularly sensitive to protease). FN-YFP sedimented at 12.3 S under low-salt conditions and at 10.0 S under high-salt conditions (Table 1), in agreement with previous studies showing that FN forms an extended conformation at a high ionic strength.26 These $S$ values are very similar to estimated values of 12.2 and 9.8 under low- and high-salt conditions, respectively, for recombinant FN without YFP in the study presented here (described below and in Table 2), indicating that the insertion of YFP into FN has a minor effect on conformation.

When we tested the deletion mutants, FNΔIII1–3 sedimented very much like FN-YFP did (Figure 3 and Table 1). Other deletion mutants, FNΔI–5 and FNΔIII1–8 (Figure 3), as well as FNΔIII11–14 and FNmono showed a small shift in sedimentation coefficient between the two salt concentrations (Table 1). FNtnA–D, in which ΔIII1–8 are replaced by seven FN domains from tenasin-C and which is also missing ΔIII11–14, showed a very minor shift, suggesting that the conformation of this chimeric molecule is not sensitive to salt concentration. These results indicate that domains within I1 and III4 are involved in forming the compact conformation under low-salt conditions, and III1–3 appears to be expendable.
FNΔ1−5, FNΔIII1−3, and FNtnA−D formed a barely detectable matrix (<8%), although small fibrils were occasionally seen by microscopy (Figure 4A).

**Co-Assembly of the FN Deletion Mutants with FN-CFP.**
In the previous experiment, the deletion mutant was the only FN present. We also tested the ability of the FN mutants to co-assemble with FN-CFP (as the wild type). As shown in Figure 5, FN-YFP nicely co-assembled with FN-CFP, and most of the mutants showed significant co-assembly. The assembly was quantitated by solubilizing the CFP and YFP in the matrix (Figure 6). Most of the mutants slightly decreased the level of the FN-CFP matrix (70−95%). In the case of FNΔ1−5, however, the level of the FN-CFP matrix was increased by ∼15%; this was the only mutant that did not co-assemble with FN-CFP. FNΔIII1−3 and FNΔIII4−8 co-assembled well with FN-CFP (70−80%). In contrast, FNΔIII11−14, FNNmono, and FNTnA−D also co-assembled, but at a reduced level (∼30−50%).

**Conformations of the GFND Mutants.**
The quality of purified GFND mutants was analyzed by SDS−PAGE (5% acrylamide) under nonreducing and reducing conditions (Figure 6).

### Table 1. Sedimentation of the FN Deletion Mutants and the FNTnA−D Chimera

<table>
<thead>
<tr>
<th>FN mutant</th>
<th>MW (kDa)a</th>
<th>S in low salt, mean (three measurements)</th>
<th>S in high salt, mean (three measurements)</th>
<th>change in S (ΔS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN-YFP</td>
<td>556</td>
<td>12.3 (12.4, 12.2, 12.4)</td>
<td>10.0 (10.0, 9.9, 10.0)</td>
<td>2.3</td>
</tr>
<tr>
<td>FNΔ1−5</td>
<td>502</td>
<td>10.7 (10.7, 10.7, 10.8)</td>
<td>9.4 (9.2, 9.5, 9.5)</td>
<td>1.3</td>
</tr>
<tr>
<td>FNΔIII1−3</td>
<td>492</td>
<td>12.1 (12.3, 12.1, 12.0)</td>
<td>9.6 (9.7, 9.5, 9.7)</td>
<td>2.5</td>
</tr>
<tr>
<td>FNΔIII4−8</td>
<td>456</td>
<td>10.9 (10.9, 11.0, 10.9)</td>
<td>9.9 (9.9, 9.8, 9.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>FNΔIII11−14</td>
<td>478</td>
<td>10.4 (10.2, 10.4, 10.5)</td>
<td>9.4 (9.2, 9.5, 9.6)</td>
<td>1.0</td>
</tr>
<tr>
<td>FNNmono</td>
<td>276</td>
<td>8.5 (8.2, 8.5, 8.8)</td>
<td>7.4 (7.1, 7.5, 7.7)</td>
<td>1.1</td>
</tr>
<tr>
<td>FNTnA−D</td>
<td>455</td>
<td>10.1 (10.0, 10.3, 10.1)</td>
<td>9.8 (9.6, 10.0, 9.9)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

aMolecular weights are deduced from amino acid sequences that do not include carbohydrates and are presented for the dimer, except for FNNmono.

### Table 2. Sedimentation of the GFND Mutants

<table>
<thead>
<tr>
<th>MW (kDa)a</th>
<th>S in low salt, mean (three measurements)</th>
<th>S in high salt, mean (three measurements)</th>
<th>change in S (ΔS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN</td>
<td>501</td>
<td>12.2 (12.4, 12.1, 12.2)</td>
<td>9.8 (9.6, 9.8, 9.9)</td>
</tr>
<tr>
<td>FN(Y973C)</td>
<td>1002b</td>
<td>13.0 (13.0, 13.2, 12.8)</td>
<td>11.2 (11.0, 11.3, 11.4)</td>
</tr>
<tr>
<td>FN(W1925R)</td>
<td>501</td>
<td>11.4 (11.4, 11.4, 11.3)</td>
<td>9.8 (9.7, 9.9, 9.8)</td>
</tr>
<tr>
<td>FN(L1974R)</td>
<td>501</td>
<td>11.3 (11.3, 11.4, 11.3)</td>
<td>9.5 (9.4, 9.5, 9.5)</td>
</tr>
</tbody>
</table>

aMolecular weights are deduced from amino acid sequences that do not include carbohydrates and are presented for the dimer, except for the Y973C mutant. bThe majority of the Y973C mutants were disulfide-bonded tetramers.

Figure 3. Glycerol gradient sedimentation of two FN deletion mutants. Fractions from the gradients were analyzed by SDS−PAGE (10% acrylamide) under nonreducing conditions. Both mutants FNΔIII1−3 and FNΔIII4−8 showed a shift in sedimentation coefficient between low and high salt concentrations. Internal standards, catalase (11.3 S), aldolase (7.3 S), bovine serum albumin (4.6 S), and ovalbumin (3.5 S), were used to calibrate the gradients. The dimeric FN mutants are seen in fractions 3−4 (low salt) and 5−6 (high salt). Monomeric fragments, seen in fractions 6−7 (low salt) and 7−8 (high salt), also shifted.

Figure 6. Most of the mutants slightly decreased the level of the FN-CFP matrix (70−95%). In the case of FNΔ1−5, however, the level of the FN-CFP matrix was increased by ∼15%; this was the only mutant that did not co-assemble with FN-CFP. FNΔIII1−3 and FNΔIII4−8 co-assembled well with FN-CFP (70−80%). In contrast, FNΔIII11−14, FNNmono, and FNTnA−D also co-assembled, but at a reduced level (∼30−50%).
The majority of the proteins were verified to be disulfide-bonded dimers. However, the Y973C mutant ran primarily as a tetramer, indicating that the cysteine residue was exposed on the surface and was able to form an additional disulfide bond. The possibility of abnormal disulfide bond formation in the Y973C mutant was postulated in the original GFND study. When we tested the GFND mutants for matrix assembly, they all formed the matrix like wild-type FN (Figure 7B). In the case of Y973C, it formed even more matrix than wild-type FN did (Figure 7B). The sedimentation coefficients for the GFND mutants were also determined in glycerol gradients. Two of the GFND mutants, W1925R and L1974R, in which the mutations were located on III13, sedimented very much like wild-type FN under high-salt conditions (Table 2). Under low-salt conditions, however, both mutants sedimented slower than recombinant FN and FN-YFP did (Table 1), indicating that these mutations somewhat compromised the compact conformation. Interestingly, the sedimentation coefficients of the Y973C mutant, 13.0 S under low-salt conditions and 11.2 S under high-salt conditions (Figure 7C and Table 2), show that the tetramers are still capable of undergoing the compact to extended conformational change.

**DISCUSSION**

**Conformation.** Johnson et al. proposed a model for the compact conformation of FN, where it is formed from an interaction between III2−3 of one subunit and III12−14 of the other subunit. The work presented here confirms the importance of III12−14 for the compact conformation. However, our deletion mutant FNΔIII1−3, which lacked domains III2−3, formed the compact conformation as well as full-length FN, suggesting that III2−3 is not an essential interaction. Recently, it...
has been reported that the binding of a bacterial adhesin to \(1^-5\) induces an extended conformation.\(^{26}\) This study confirmed that \(1^-5\) contributes to the compact conformation, because deleting it reduced the magnitude of the change. Deletion of \(1^-4^-8\) or \(11^-14\) had stronger effects, but all of these deletion mutants still showed a conformational change. This suggests that there are multiple interactions involved in forming the compact conformation. The existence of multiple FN conformations has been proposed previously on the basis of a monoclonal antibody that was sensitive to conformation.\(^{6,10}\)

In our sedimentation analysis, FNmono showed a change in sedimentation from low- to high-salt conditions (Table 1). Sedimentation shifts were also seen in the proteolytic fragments of FN\(\Delta1^-3\) and FN\(\Delta4^-8\), which were presumably monomeric (Figure 3). This could be due to a conformational change within the monomer as suggested by Erickson and Carrell,\(^{5}\) or it could be due to dimerization of the monomer under low-salt conditions as reported by Johnson et al.\(^{16}\) For the FN dimer, however, the sedimentation shift is probably due to binding of one subunit to its partner, accommodated by bending at hinge points and resulting in the compact conformation. On the basis of the sedimentation coefficients of the FN monomer and FN dimer under low-salt conditions, a dimeric form produced by association of two FN monomers is unlikely to be as compact as the disulfide-bonded FN dimer.

**Assembly.** The laboratories of Schwarzbauer\(^{27^-29}\) and Sekiguchi\(^{30^-32}\) pioneered the study of FN assembly by using FN deletion mutants. Most of our results are consistent with their earlier findings. Both agree that \(1^-5\) is crucial for assembly, and we now show additionally that FN lacking \(1^-5\) cannot even co-assemble with full-length FN. In addition to \(1^-5, 31^-3\) also appears to be important for matrix assembly, because the mutants lacking these domains (FN\(\Delta1^-3\) and FN\(\Delta1^-5\)) did not form a matrix. This is consistent with the earlier study of Sechler et al.\(^{29}\) who showed that deletion of \(31^-2\) or \(32^-5\) substantially compromised matrix assembly. They also showed that deletion of \(2^-1\) did not affect assembly, strongly implicating \(32\) as the important domain.

Domain \(32\) is now a prime candidate for an essential role in matrix assembly, perhaps by binding the other essential matrix assembly site, \(1^-5\). We showed previously that \(32\) could bind \(1^-5\) in solution, but only when \(32\) was destabilized by removing the G strand. Sechler et al.\(^{29}\) showed that \(32\) coated on plastic could bind whole FN or the 70 kDa fragment (\(1^-5\)). The adsorption to plastic may have partially denatured \(32\), exposing the binding site for \(1^-5\). We also found previously that introducing a disulfide bond in \(32\), which stabilized folding, significantly reduced the level of the FN matrix.\(^{16}\) The binding of the N-terminal region, \(1^-5\) to \(32\) also agrees with the N-terminal 30-40 nm overlap between FN molecules in matrix fibrils observed by super-resolution microscopy.\(^{33}\)

All constructs that contained a \(1^-5\) segment could co-assemble with wild-type FN. This suggests that the primary event in matrix assembly is binding of \(1^-5\) on the soluble FN to an FNIII domain in the already assembled matrix, but this binding event apparently cannot go in the opposite direction. For instance, \(1^-5\) from wild-type FN already incorporated in the matrix cannot bind FNIII domains on soluble FN\(\Delta1^-5\). This is consistent with the idea of cryptic binding sites that are exposed only in matrix fibrils under tension.\(^{34,35}\) Specifically, the FNIII sites that bind \(1^-5\) are inactive in soluble FN dimers and are activated by a conformational change and/or domain unfolding, induced by tension or another mechanism, when FN is incorporated into matrix fibrils.

**Fibronectin Glomerulopathy (GFND).** Castelletti et al.\(^{17}\) generated recombinant heparin binding fragments containing \(12^-14\) with GFND mutations (W1925R or L1974R) in FN\(\Delta1^-3\) and found that these mutations significantly reduced the level of heparin binding. Both mutations substitute buried hydrophobic core residues with arginine. Therefore, these mutants are likely to have folding problems. The other mutation, Y973C in \(2^-1\), was not characterized in the original report. The tyrosine residue is at the beginning of the F strand and is highly conserved in FNIII domains. It helps stabilize the domain by forming a tyrosine corner,\(^{56}\) and the corresponding tyrosine residues in crystallized FNIII domains\(^{37,38}\) are mostly buried. Our study shows that the Y973C mutant forms additional disulfide bonds, indicating that the residue is exposed on the surface by misfolding. When we tested the conformations of the full-length GFND mutants, the W1925R and L1974R mutants formed slightly less of the compact conformation under low-salt conditions. The mutations did not affect matrix assembly dramatically. Unfortunately, these results do not provide any direct explanation for how these mutations cause GFND.

The originally reported GFND mutations from patients were heterozygous missense mutations, while our recombinant GFND mutants are homogeneous dimers. Therefore, the GFND mutations in patients should have milder effects on conformation. In addition, there is no evidence indicating that the GFND mutations cause thrombosis by misfolding of FN in patients. We previously reported that the folding and stability of FNIII domains were crucial for anastellin-induced FN aggregation.\(^{15,16}\) We suggest that the GFND mutations destabilize the FNIII domains and these unstable FNIII domains
may slowly form nonfibrillar aggregates in the kidney. In the case of the Y973C mutant, it is also possible that unusual disulfide bond formation, even in the case of a heterozygous mutation, enhances nonfibrillar aggregation.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS

FN, fibronectin; GFND, glomerulopathy with fibronectin deposits; FNIII, fibronectin type III; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; CCD, charge-coupled device.

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