Fibronectin (FN) is a secreted protein expressed in the liver and circulated in blood as a soluble dimer (1). In addition to this plasma FN, some cell types, such as fibroblasts and endothelial cells, secrete FN and assemble it into extracellular matrix fibrils. FN is a modular protein containing 12 FN type I (FN1) domains, two FN type II domains, and 15–17 FN type III (FNIII) domains (2, 3). Two cysteines at the C terminus asymmetrically form interchain disulfide bonds (4, 5). Dimeric FN molecules interact with each other on the cell surface to form matrix fibrils. This process requires cell surface integrins (6–9). Live cell imaging showed that integrin translocation by the actin cytoskeleton is crucial for the early stages of FN matrix assembly (10, 11). More recently, it has been reported that cell-to-cell adhesion via cadherin controls tissue tension and affects FN matrix formation during embryogenesis (12).

The FN matrix had long been thought to be formed by disulfide-bonded FN multimers because the protein migrated at the top of an SDS gel under nonreducing conditions (13–16). However, studies by Chen and Mosher (17) and by our laboratory (18) demonstrated that the FN matrix was composed of FN dimers that are further cross-linked by noncovalent bonds, whose nature is not known. For the past three decades, FN matrix assembly has been studied with FN fragments, antibodies, and deletion mutants. These studies indicate that several domains are crucial for FN matrix formation: I1–9 (19–24), III1 and/or III2 (20, 25, 26), III4–5 (27), III7–10 (20, 23, 24), III12–14 (28), the variable domain (8), I10–12 (29), and the interchain disulfide bonds at the C terminus involved in dimerization (20). III7–10 and the variable domain seem to play roles as integrin binding sites. III4–5, III12–14, and I10–12 have contradictory reports (24, 26, 30), so they may only be required for particular cell types or under some conditions. On the other hand, many studies consistently show that I1–9 is crucial for FN matrix assembly. More precisely, I1–5 appears to be important because an antibody against this region inhibited FN matrix assembly (23), and mutants in which this region was deleted did not form the FN matrix (20).

In addition to I1–9, many studies indicate that III1 and/or III2 are important for FN matrix assembly. An antibody that recognized III1 was able to inhibit FN matrix formation (25). Solid phase binding assays showed that III1–2 and III2 (without the linker between III1 and III2), when adsorbed to plastic, interacted with full-length FN and I1–9 (26, 31). Sechler et al. (26) showed that FN deletion mutants lacking III2 had significantly decreased matrix assembly.

A recent NMR study showed a unique structure for the domain pair III1–2 (32). It had been recognized from sequence analysis that there is an 18-amino acid linker between these domains. The NMR structure showed that the A strand of III2 was disordered, giving a total length of ~35 amino acids for the linker, and further indicated that III1 and III2 formed a closed compact structure, with a potential salt bridge between Lys-669 in III1 and Asp-767 in III2. These two amino acids were mutated to alanine, giving the mutant designated KADA. The native III1–2 was found not to bind I1–5, but the KADA mutation was reported to dramatically enhance binding of I1–5, as measured by surface plasmon resonance. The authors proposed that
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III1–2 had a cryptic high affinity binding site for 1–5, which could be exposed when the KADA mutations disrupted the binding of III1 to III2.

Another study used fluorescent protein (FP)-based FRET to explore the conformation of III1–2 (33). It was reported elsewhere that a construct with two FNII 1 domains (III7–8) inserted between YFP and CFP showed a negligible FRET signal (34). In contrast, when III1–2 was inserted between the FPs, there was a significant FRET signal, consistent with the model that the two domains folded back over the flexible linker and made contact, bringing the N and C termini close together (33). Surprisingly, however, in that study the KADA mutations increased the FRET signal rather than decreasing it. The addition of 1–9 reduced the FRET signal of the KADA mutant and had no effect on the FRET of wild type. These authors also suggested a cryptic binding site for 1–9 in III1–2, which was exposed in the KADA mutant.

In contrast to many protein polymers, which can self-assemble in vitro, FN matrix cannot be reconstructed in a test tube. However, there are several approaches to make artificial FN aggregates that resemble the matrix. One of the methods uses anastellin, a truncated form of the first FNIII domain, lacking the A and B β strands (35, 36). Anastellin binds FN to form a large aggregate, called superFN, which has FN matrix-like fibrillar structures when observed by light microscopy. Our previous studies indicated that the opening or unfolding of FNIII domains is important for anastellin binding and aggregation (24, 37). We also found that the N-terminal FN fragment, 1–9, which prevented FN matrix formation in cell culture, did not inhibit superFN aggregation. Instead, 1–9 co-precipitated with the aggregate (24). In the present study, we further investigated the mechanism of superFN aggregation and FN matrix assembly, by introducing disulfide bonds that prevented opening of the targeted FNIII domains.

**EXPERIMENTAL PROCEDURES**

**Bacterial Protein Expression**—The cDNA fragments of III1–2, III1–2(4aa) (which has a four-amino acid extension at the C terminus), III1–2ΔL (a 14-amino acid deletion in the linker), III12, LIII12 (with the linker), III2ΔA (the A strand deletion), and III2ΔG (the G strand deletion) were amplified by PCR using the original construct as a template and were cloned into the pET15b expression vector (Novagen), which added an N-terminal His tag. The additional N-terminal sequence is MGSSHHHHH-HHSSGLVPRGSHM. Fragments III1 (SGPV... FTTT), III1–2 (SGPV... SQTT), III1–2(4aa) (SGPV... SQTTAPDA), III1–2ΔL (SGPV... SQTT without PVTSNTVGETTPF), LIII12 (TTST... APDA), III12 (SPLV... SQTT), III2ΔA (TASS... SQTT), III2ΔG (SPLV... GEQS), and III11 (EIDK... TAVT) were expressed in *Escherichia coli* BL21 (DE3) at 37 °C and were purified with a cobalt column (TALON; Clontech) using standard procedures. Proteins were eluted with imidazole from the column and were dialyzed against 20 mM Tris with 150 mM NaCl (TBS, pH 8.0) or PBS (pH 7.2). Most of the proteins were obtained from the soluble portion of the bacterial lysate except for III2ΔG, which required denaturation and renaturation, using the procedure we reported previously for anastellin (24). Previously generated III1L and anastellin were also used in this study (24). Disulfide mutations in III1SS (S625C and K669C), III2SS (S753C and P778C), and III1SS (S1648C and E1691C) were generated by site-directed mutagenesis with *Pfu* Turbo DNA polymerase (Stratagene), like III3SS engineered previously (37). Disulfide bonds are in equivalent positions in each of the domains as shown in Fig. 1. We also created III1–2KADA (K669A and D767A), III1SS-2, and III1–2SS mutants by mutagenesis. The KADA mutations were verified by MALDI-TOF mass spectrometry in addition to DNA sequencing. The amino acid numbering used here follows that of recent publications (32, 33), in which the N-terminal 31-amino acid pre (signal)-pro (furin-recognition) sequence is included. In addition to the FN fragments, we made the functional upstream domain (FUD; from *Streptococcus pyogenes*) construct. The original FUD construct (38) was kindly provided by Drs. Bianca Tomasini-Johansson and Deane Mosher at the University of Wisconsin. Because FUD is a small peptide of 49 amino acids, even with a His tag it was difficult to see in SDS-PAGE at low concentrations. Therefore, we fused FUD to maltose-binding protein (MBP) for visualization. The cDNA fragment of FUD was amplified by PCR using the original construct as a template and was cloned into the author-made MBP expression vector, in which MBP was inserted into the pET24b vector (Novagen). The sequence of the MBP construct is MKIE... (MBP)... IKTaaaLEHHHHHH (the MBP sequence is underlined). The sequence of the MBP-FUD construct is MKIE... (MBP)... IKTaaaKDQ... (FUD)... TEDTaalaLEHHHHHH. These constructs were expressed and purified with standard procedures the same as most of the FN fragments mentioned above. The concentrations of purified proteins were determined from their absorbance at 280 nm using the extinction coefficient of each protein calculated by the Protean computer program (DNAstar Inc.). 5,5'-Dithio-bis (2-nitrobenzoic acid) was used to confirm disulfide bond formation under denaturing conditions (37, 39). SDS-PAGE was performed with standard procedures.

We also created FP-based FRET constructs in which PCR-amplified III1–2, III1–2KADA, III2 and III2ΔG were cloned into the SpeI and KpnI sites between mYPet and mECFP*. Each FP has a monomeric mutation, A206K (34). The sequence of the FRET construct is MGSSHHHHH-HHSSGLVPRGSHMggrM-VSK... (mYPet)... ELYKts (III1–2, III1–2KADA, III2, or III2ΔG)gMVSK... (mECFP*)... ELYKggr (the mYPet and mECFP* sequences are underlined). These FRET constructs were expressed in *E. coli* C41 (DE3) at room temperature and purified as described above. The concentrations of the FRET constructs were estimated from the absorbance at 433 nm using the extinction coefficient of mECFP* (23,500 m⁻² cm⁻¹).

**Mammalian Cell Protein Expression**—For transient mammalian cell expression, the cDNA fragments of 1–9 (QAQQ... TTSS) and 1–5 (QAQQ... YPSS) were amplified by PCR with FN-YPet/Neo (18) as a template and were cloned into the pHLSec2 vector, which was slightly modified from the original pHLSec vector (40) by Dan Leahy (Johns Hopkins University). This vector has an engineered signal sequence, which gives an additional three amino acids (EGS) at the N terminus of the secreted protein and also adds a C-terminal His tag (EFHHHHHHHHH). FN-YPet (QAQQ... DSRE, where monomeric YPet
is inserted between III6 and III7) was also cloned into the pHLSec2 vector without adding the C-terminal His tag. The full-length FN disulfide mutants, FN2SS, FN3SS, FN3–11SS, and FN2–3-11SS, were generated by site-directed mutagenesis of FN-YPet. Purified expression vectors were transfected into HEK293T cells with polyethylenimine (25-kDa branched; Aldrich) as reported previously (40). To avoid exogenous FN contamination from serum, the serum-free medium Hybridoma SFM (Invitrogen) was used for transfection and expression. HEK293T produces a negligible amount of endogenous FN. The conditioned medium was collected after 4–6 days of transfection. I1–9 and I6–9 were purified with a cobalt column using imidazole, whereas FN-YPet and the disulfide mutants were purified with a gelatin column using 4M urea for elution. To exchange the buffer, purified proteins were run through a PD-10 column (Amersham Biosciences), which was equilibrated with TBS or PBS. We also tried to express I6–9, but it suffered from proteolysis during expression. Because I1–9, I6–9, and I1–5 fragments and anastellin have a C-terminal His tag. The rest of the FN fragments have an N-terminal His tag. I11 and I12 are I11 and I12 with the 18-amino acid linker. I1–2KADA is I1–2 with K669A and D767A mutations. I1–2(4aa) and I1–2KADA(4aa) have an extra four amino acids at the C termini. I1SS, I1SS-2, I2SS, I3SS, I4SS, and I5SS have engineered disulfide bonds. I1–2ΔA is I1–2 with the 14 amino acids of the linker deleted. I2ΔA is I2 with the A strand deleted, and I2ΔG is I2 with the G strand deleted. The tryptophan and engineered disulfide bond are indicated in the I2SS structure, which was created by the PyMol computer program (Schrödinger) after modeling the disulfide mutations with SWISS-MODEL.

Pelleting Assay—The proteins of interest were mixed in a final volume of 25 μl with TBS or PBS containing 5 mM EDTA and incubated at room temperature for ~16 h. After incubation, the samples were centrifuged at 20,000 × g for 10 min, and the supernatants were collected. The pellets were rinsed with 100 μl of TBS or PBS and resuspended with SDS-PAGE loading buffer. SDS-PAGE (15%) was performed using standard procedures.

Fluorescence Measurements—Fluorescence measurements were performed with a Shimadzu RF-5301-PC spectrofluorometer (37). For intrinsic tryptophan fluorescence measurements, 1 μM purified single FNIII domains in PBS were excited at 280 nm in the presence of 0–8 M urea, and emission spectra were recorded at 1-nm intervals from 300 to 400 nm with slit widths of 3 nm for excitation and 5 nm for emission. The measurements for the disulfide mutants were performed with or without 1 mM DTT.

For FRET measurements, 1 μM samples were excited at 433 nm, and emission spectra were recorded at 1-nm intervals from 440 to 620 nm using slit widths of 3 nm for both excitation and emission. These measurements were done with quadruplicate
samples at room temperature. Trypsin (10 μg/ml) digestion and I1–9 (2 μM) incubation were carried out for 1 h before measurements were made.

Gelatin Precipitation Assay—The proteins of interests were mixed in a final volume of 200 μl of PBS containing 5 mM EDTA and incubated for 1 h at room temperature. 30 μl of gelatin-
agarose (Sigma) was added and incubated for an additional hour at room temperature. After incubation, the samples were centrifuged at 300 rpm for 1 min, and the supernatants were removed. The agarose beads were washed with 1 ml of PBS five times. The bound components were eluted from the beads with 30 mM H9262 urea/PBS.

**Glycerol Gradient Sedimentation and Gel Filtration Chromatography**—We used glycerol gradient sedimentation to estimate the sedimentation coefficients. Wild-type III1–2 and III1–2KADA were sedimented on a 15–40% glycerol gradient in 0.2 M ammonium bicarbonate at 50,000 rpm for 16 h with a Beckman SW 55.1 rotor. Aldolase (7.3 S), BSA (4.6 S), ovalbumin (3.5 S), and cytochrome c (1.7 S) were used to calibrate the glycerol gradients.

The Stokes radii ($R_s$) of III1–2 and III1–2KADA were estimated by gel filtration on a Sephacryl S-100 column (GE Healthcare) at a flow rate of 1 ml/min. PBS was used for elution. The column was calibrated with BSA ($R_s = 3.55$ nm), ovalbumin (3.05 nm), and cytochrome c (1.41 nm).

**Cell Culture and FN-YPet Quantification Using YPet Fluorescence**—The FN(–/–) cell line SF was kindly provided by Deane Mosher (University of Wisconsin). These cells were maintained with DMEM containing 10% FCS (Sigma). The cells were harvested with 0.05% trypsin, 2 mM EDTA (Invitrogen), after which 2 mM PMSF was added to quench the trypsin. The cells were rinsed with DMEM and resuspended with DMEM containing 1% FCS, which was depleted of FN by passage through a gelatin column. Cell suspensions (0.4 ml; $10^5$ cells/ml) containing 30 nM FN-YPet with various FN fragments or FN2SS, FN3–11SS, or FN2–3-11SS were plated into a 24-well culture plate. The concentrations of FN-YPet and disulfide mutants were estimated from the absorbance at 514 nm with the molar extinction coefficient of monomeric YPet, $85,000 M^{-1} cm^{-1}$ (34). 12-mm circular coverglasses were added to several wells for microscopy. After the cells were cultured for ~16 h, each well was rinsed three times with 0.5 ml of PBS containing Ca$^{2+}$ and Mg$^{2+}$ and then treated with 0.1 ml of trypsin (10 μg/ml) in PBS containing 5 mM EDTA at 30 min at room temperature. Trypsin digestion was quenched with 2 mM PMSF, and samples were centrifuged at 15,000 rpm for 10 min to remove the cells. This solubilized most of the matrix, as indicated by the loss of almost all fluorescence in the light microscope. Total matrix FN-YPet was then determined from fluorescence of the supernatant. Samples were excited at 514 nm,
and emission spectra were recorded at 528 nm using slit widths of 3 nm for excitation and 5 nm for emission. These measurements were carried out with quadruplicate samples at room temperature. Note that mild trypsin digestion does not affect YPet fluorescence (34). For microscopy, the samples were fixed with 3.7% formaldehyde in PBS, washed, and mounted on slides. The samples were observed with a light microscope (Zeiss Axiophot), and the images were captured with a cooled charge-coupled device camera (CoolSNAPHQ; Roper Scientific).

RESULTS

SuperFN-like Aggregation from III1–2: Search for Blocking Mutants—The recombinant proteins used in this study are diagrammed in Fig. 1. We have previously shown that a disulfide bond introduced into III3, locking the B strand to the E strand, inhibited anastellin binding (37). Here we have introduced the equivalent disulfide bond into domains III1, III2 (Fig. 1), and III11. We have also previously shown that the whole FN molecule is not needed for superFN formation; a small FN fragment, III1–2, forms a superFN-like aggregate when mixed with anastellin (24). In addition to these disulfide locks, we have investigated mutants III1–2KADA, and III1–2L, in which 14 amino acids were deleted from the 18-amino acid linker between III1 and III2. Two additional mutants, III1–2/4aa and III1–2KADA/4aa, have a four-amino acid extension (the first four amino acids of III3) at the C terminus of III2, which significantly

FIGURE 5. ‘1–9 co-precipitates with the superFN-like aggregates. A, ‘1–9 co-precipitates with III1–2 mutants and anastellin. 10 μM III1–2 mutants plus 2 μM ‘1–9 (70 kDa) or BSA were mixed with 40 μM anastellin. ‘1–9 was able to co-precipitate with all the III1–2 mutants/anastellin aggregates. BSA remained in the supernatant. S, supernatant; P, pellet; Std, BenchMark protein ladder (Invitrogen). B, FUD (bacterial adhesin) inhibits ‘1–9 co-precipitation with the aggregate. 10 μM III1–2 and 2 μM ‘1–9 were mixed with 40 μM anastellin. 2 μM MBP or MBP-FUD was also added to the reaction. MBP-FUD inhibited ‘1–9 co-precipitation with the III1–2/anastellin aggregate, but MBP did not.

FIGURE 6. Activities of ‘1–9, ‘6–9, and ‘1–5 for binding the III1–2/anastellin aggregate and inhibiting FN matrix formation. A, for the pelleting assay, 2 μM ‘1–9, ‘6–9 (40 kDa), or ‘1–5 (30 kDa) was mixed with 40 μM anastellin, with or without 10 μM III1–2. A significant amount of ‘1–9 co-precipitated with the III1–2/anastellin aggregate. On the other hand, only a trace amount of ‘1–5 co-precipitated, and ‘6–9 did not co-precipitate at all. None of the fragments formed any aggregates with anastellin in the absence of III1–2. S, supernatant; P, pellet; Std, BenchMark protein ladder (Invitrogen). B, various FN fragments (1 μl) were added to FN(“+”) cell culture with FN-YPet (30 nM) and cultured for 16 h. ‘1–9 inhibited FN matrix formation, ‘6–9 had no significant effect on FN matrix assembly, and ‘1–5 reduced the FN matrix. C, the amount of matrix FN was estimated by measuring YPet fluorescence after solubilizing YPet from cell culture by trypsin treatment. The emission intensity was normalized to that of a control sample (no fragment). ‘1–9 and ‘1–5 reduced the FN matrix by 50 and 20%, respectively. The error bars indicate standard deviation. *, p < 0.01.
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improved the solubility of these proteins. Similar C-terminal extensions are known to stabilize other FNIII domains (41, 42).

We tested these constructs for ability to assemble a superFN-like aggregate, using a pelleting assay (Fig. 2). Our preliminary study indicated that 40 \( \mu \)M anastellin was required to efficiently precipitate 10 \( \mu \)M III1–2 (supplemental Figs. S1 and S2). The four-amino acid extension on the C terminus of III1–2 completely suppressed superFN-like aggregation, suggesting that the stability of III2 is a key factor in aggregation. In full-length FN, those four amino acids are probably incorporated into III3 and thus would not lead to stabilization of III2. In fact, in our previous study, fragments containing both III2 and III3 (III1–3) were able to form superFN-like aggregates (24). The KADA mutations had no effect on aggregation. The disulfide lock in III1 had no effect, whereas the disulfide in III2 completely blocked aggregation. Removing the linker had no effect. Overall, these data are consistent with the interpretation that instability and opening of III2 is essential for superFN-like aggregation of III1–2.

We then tested the stability of FNIII domains III1, III2, and III1 to chemical denaturation. III1 was included because it was previously found to bind anastellin (24). Intrinsic tryptophan fluorescence was used to examine the stability of the single FNIII domains in the presence of urea. All FNIII domains of FN have a conserved buried tryptophan as shown in Fig. 1. III1 has two additional tryptophans, one of which is exposed on the surface. A broad emission peak for III1, centered near 330 nm under physiological conditions, was shifted toward 350 nm at urea concentrations 7 and 8 M (Fig. 3). The emission peak for III2 near 310 nm was shifted toward 350 nm, and the intensity was increased at urea concentrations above 5 M. The emission peak for III1 near 310 nm was shifted toward 350 nm, and the intensity was increased at urea concentrations above 1 M. III1 is the weakest domain among the FNIII domains we have tested. In contrast, the disulfide mutants, III1SS and III2SS, were quite stable against urea denaturation (Fig. 3), as previously reported for III3SS (37). III1SS was also stabilized by the disulfide bond relative to wild-type III1, although it was still denatured by 6 M urea.

Anastellin binding to these FNIII domains was analyzed by ANS fluorescence. We previously found that the reactivity of ANS to anastellin was reduced when anastellin formed a complex (37). The emission intensity of ANS with FNIII domains alone was negligible (Fig. 4), consistent with these domains being folded properly with no exposed hydrophobic patches that could interact with ANS. On the other hand, the emission intensity of ANS was dramatically increased when added to anastellin, with an emission maximum of \( \sim 470 \) nm. In the presence of III2 or III1, the emission intensity was significantly reduced (Fig. 4), whereas III1, III1SS, III2SS, and III1SS had no effect, indicating that there was no interaction with anastellin. In the presence of DTT, however, III2SS and III1SS were able to reduce the emission intensity like wild-type III2 and III1.

Binding of I1–9 to III1–2—Because I1–9, which is one of the matrix assembly sites, interacted with superFN (24), we examined whether I1–9 can bind the III1–2/anastellin aggregate. Indeed, I1–9 was co-precipitated not only with the wild-type III1–2 aggregate but also with III1–2KADA, III1SS-2, and III1–2L mutants (Fig. 5A). These results suggest that the presence of the linker or unfolding of III1 does not contribute to I1–9 binding. The specificity of I1–9 binding to the aggregate was also confirmed by inhibition with FUD (Fig. 5B), a 49-amino acid peptide from a bacterial adhesin that is known to bind I1–9 with high affinity (43, 44).
We also tested the two smaller fragments of I1–9, I1–5 and I6–9, for the ability to bind the III1–2/anastellin aggregate (Fig. 6A). Only a trace amount of I1–5 co-precipitated, whereas I6–9 did not co-precipitate at all. We then tested the ability of I1–9 and its two fragments to inhibit FN matrix assembly in cell culture. Fluorescence microscopy showed that I1–9 inhibited FN matrix formation as reported previously (19, 23, 24). The partial inhibition of matrix assembly in the present study is less than the almost complete inhibition in our previous study (24), probably because of the different cell type and culture conditions, including the lower concentration of I1–9. I1–5 reduced the matrix, whereas I6–9 had no effect on FN matrix assembly (Fig. 6B).

To quantitate the matrix, we measured the YFP fluorescence released into the soluble fraction after trypsin treatment (Fig. 6C and supplemental Fig. S3). I1–9 reduced the FN matrix by 50%, I6–9 had no significant effect on FN matrix assembly, and I1–5 decreased the matrix by 20%. These results showed that the longer segment, I1–9, has stronger binding to the superFN-like aggregate and a more inhibitory effect on FN matrix formation than either of the shorter fragments.

Because I1–9 is capable of binding gelatin through the I6–9 region (45), we used a gelatin precipitation assay to study the interaction between I1–9 and several FN fragments. Before using this assay, we verified that gelatin (45%) had no effect on FN matrix formation when added to cell culture. An earlier study showed that fluorescently labeled gelatin is able to stain FN matrix fibrils (46), indicating that the gelatin binding sites are not blocked by matrix assembly. In the gelatin precipitation assay, III1–2 and its mutants did not co-precipitate with I1–9 (Fig. 7A). This was surprising because III1–2KADA was previously reported to bind I1–5 and I1–9 (32, 33). It is possible that gelatin binding blocks the interaction between I1–9 and III1–2KADA. We also added III2/H9004G to cell culture, but it had no effect on FN matrix formation (Fig. 7, B and C, and supplemental Fig. S3). The only mutant that showed any binding to I1–9 was III2/H9004G, which is discussed below.

### Structural analysis of III1–2 and the KADA Mutant

The previously studied KADA mutant was reported to run far ahead of the wild-type III1–2 on gel filtration and was thought to have a larger hydrodynamic radius (32). We therefore examined our III1–2KADA by glycerol gradient sedimentation and gel filtration chromatography. Both wild-type III1–2 and III1–2KADA eluted at the same Rs, 2.5 nm (data not shown).

We also examined the difference between III1–2 and III1–2KADA using FRET, repeating the analysis of Karuri et al. (33). Our FRET construct, FRET(III1–2), had mYPet and mECFP* on the N and C termini of III1–2, as diagramed in Fig. 8A. The emission spectra of FRET(III1–2) with and without trypsin digestion are shown in Fig. 8A. The FRET signals were determined by FRET efficiency, which was calculated from the decreased donor emission at 475 nm and by the ratio of the acceptor emission at 528 nm to the donor emission at 475 nm (Table 1). Based on FRET efficiency and the Förster distance for this fluorophore pair (5.3 nm), we estimated the distance between the two fluorophores in the FRET(III1–2) construct to

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**Table 1**

| FRET signals of III1–2, III2, and mutants | FRET efficiency | Emission ratio |
|-------------------------------------------|-----------------|----------------|
| III1–2                                    | 0.33            | 1.55           |
| III1–2KADA                                 | 0.33            | 1.56           |
| III2                                      | 0.44            | 2.23           |
| III2ΔG                                    | 0.41            | 2.23           |
| III2ΔG + I1–9                              | 0.15            | 1.09           |
| III2ΔG + I1–9 + MBP                        | 0.14            | 1.07           |
| III2ΔG + I1–9 + MBP-FUD                    | 0.42            | 2.22           |

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**Figure 8.** Diagrams and emission spectra of the FRET constructs. The C-terminal 11-amino acid segment of YFP and the N-terminal 5-amino acid segment of CFP are presumably unstructured (34). The N-terminal 17-amino acid region of III2 and the 18-amino acid linker between III1 and III2 are also unstructured. A and B, samples with and without trypsin treatment were excited at 433 nm, and emission intensity was recorded at wavelengths from 440 to 620 nm. C, addition of I1–9 to FRET(III2ΔG) significantly reduced the FRET signal (this is not a trypsin treatment, but a comparison of spectra with and without I1–9). aa, amino acids.

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We also tested the two smaller fragments of I1–9, I1–5 and I6–9, for the ability to bind the III1–2/anastellin aggregate (Fig. 6A). Only a trace amount of I1–5 co-precipitated, whereas I6–9 did not co-precipitate at all. We then tested the ability of I1–9 and its two fragments to inhibit FN matrix assembly in cell culture. Fluorescence microscopy showed that I1–9 inhibited FN matrix formation as reported previously (19, 23, 24). The partial inhibition of matrix assembly in the present study is less than the almost complete inhibition in our previous study (24), probably because of the different cell type and culture conditions, including the lower concentration of I1–9. I1–5 reduced the matrix, whereas I6–9 had no effect on FN matrix assembly (Fig. 6B).
As expected from our hydrodynamics analysis, the signal of the FRET(FII1–2KADA) construct was identical to that of wild type (Table 1). These measurements were performed with PBS. We also tested Tris buffer (pH 7.4) and different salt concentrations (0, 0.2, and 0.5 M NaCl). There was no significant difference between the wild type and KADA mutant in any of these buffer conditions. High salt reduced the FRET signal for both constructs, but we have seen this effect in other FP-based FRET constructs, perhaps because high salt induces an extended conformation of the unstructured segment. The addition of 1–9 to either the FRET(FII1–2) or FRET(FII1–2KADA) construct had no effect on the FRET signals. Thus, we did not find any difference between wild type and the KADA mutant.

A Binding Site for I1–9 in Truncated II2AG—We also tested several small FN fragments (II1L, LII2, II1, II2) and anastellin in the gelatin precipitation assay. None of the fragments bound I1–9. Therefore, we postulated that a cryptic binding site for I1–9 in the II1–2/anastellin aggregate is located on II2 and only exposed when it unfolds to form the aggregate. To further explore this hypothesis, we generated destabilized II2 mutants by deleting the N- or C-terminal b strand. The strand A deletion (III2DA) showed a spectrum similar to that of wild type in our tryptophan fluorescence assay (supplemental Fig. S5). This is consistent with a recent NMR study showing that the residues that make up strand A are unstructured (Fig. 1) (32) and thus not likely to affect folding. In the gelatin precipitation assay, III2DA also did not interact with I1–9. In contrast, in the strand G deletion (III2DG), the broad tryptophan emission peak, which was ~340 nm in the absence of urea (versus 310 nm for III2 and III2DA), was shifted to 350 nm, and the intensity was increased even in 2 M urea (supplemental Fig. S5). These results indicate that the tryptophan in this mutant is more exposed to the solvent in 0 M urea and is easily denatured by mild urea treatment. Thus, III2DG appeared to be the destabilized mutant we wanted. In the gelatin precipitation assay, III2DG co-precipitated with I1–9 (Fig. 7A), suggesting that the binding site for I1–9 is exposed within this truncated II2 domain. III2DG did not co-precipitate with II6–9.

We further studied the interaction between III2DG and I1–9 using FRET. The control construct, FRET(II2), showed a strong FRET signal (Fig. 8B and Table 1), and FRET(III2DG) was similar to wild type. Both constructs have a flexible region of ~37 amino acids in addition to the folded domain (17 amino acids for the unstructured A strand, 16 amino acids for the flexible regions of the FPs, plus four amino acids from cloning sites (see Ref. 34 for details of this calculation). The longer flexible region might bring the two FPs close together, because these FRET signals were higher than those we measured previously for inserts of single FNIII domains II6 and II10 (34). The separation of the two fluorophores was estimated to be ~5.5 nm. This distance fits to that of intrinsically unstructured peptides with similar lengths (34). The addition of I1–9 to the FRET(II2) construct had no effect on the FRET signal. In contrast, the signal of FRET(III2DG) was greatly decreased by the addition of I1–9 (Fig. 8C and Table 1), suggesting that I1–9 binding induced an extended conformation of III2DG. I1–9 binding to the FRET(III2DG) construct was inhibited by FUD (Table 1). When III2DG was tested in cell culture, it was able to inhibit FN matrix

be ~6.0 nm. This is a reasonable value, because this construct contains flexible regions of ~55 amino acids in addition to the folded domains (see our previous study for details on distance estimation for intrinsically unstructured peptides from FRET efficiency (34). Our FRET signal was higher than that of Karuri’s study (33). We expected that our FRET signal might be lower, because Karuri et al. used earlier versions of CFP and YFP that have a weak dimerizing activity, whereas we used monomeric versions (34). Their lower FRET might be attributed to their EYFP having a lower absorption or greater chloride sensitivity than the mYPet we used.

FIGURE 9. Effects of engineered disulfide bonds in full-length FN on superFN aggregation and cellular matrix assembly. A, 1 mM full-length FN-YPet (~280 kDa) or FN-YPet with the indicated disulfide mutations was mixed with 20 mM anastellin. FN2SS and FN3SS slightly reduced the aggregates. Disulfide bonds in both II3 and II11 (FN3–11SS) decreased the aggregate. Three disulfide bonds in II2, II3, and II11 (FN2–3-11SS) further reduced the aggregate. S, supernatant; P, pellet; Std, BenchMark protein ladder (Invitrogen). B, purified 30 mM FN-YPet, FN2SS, FN3–11SS, and FN2–3-11SS were added to FN(17) cell culture and cultured for ~16 h. Fluorescence microscopy showed that FN2SS and FN2–3-11SS assembled less FN matrix. C, the amount of matrix FN was estimated by measuring YPet fluorescence after solubilizing YPet from cell culture by trypsin treatment. FN2SS and FN2–3-11SS reduced the FN matrix by 20% relative to wild type. This suggests that FN2SS alone is the primary cause of the inhibitory effect. The error bars indicate the standard deviation. *, p < 0.01.
formation by ∼40% (Fig. 7, B and C, and supplemental Fig. S3), almost as much as the ∼50% inhibition by I1–9 (Fig. 6C).

**Disulfide Locks Partially Disrupt FN Matrix Assembly in Cell Culture**—Based on our present and previous results, the unfolding of FNIII domains appears to be crucial for aggregation and assembly. Therefore, we engineered several disulfide mutations in full-length FN to test our findings. The qualities of purified proteins are shown in supplemental Fig. S6. Surprisingly, the single disulfide bond in III2 (FN2SS) or III3 (FN3SS) had only a slight effect on superFN aggregation (Fig. 9A). Two disulfide bonds, in III3 and III11 (FN3–11SS), decreased the aggregate a bit more, whereas three disulfide bonds, in III2, III3, and III11 (FN2–3–11SS), greatly reduced aggregation but did not inhibit it completely. In the aggregate formed by FN2–3–11SS, the amount of anastellin was greatly reduced, indicating that the disulfides decrease anastellin binding. So how does the triple mutant even form the aggregate? It may have another anastellin binding site, or the disulfide locks may not block anastellin binding completely. In any case, these results suggest that the unfolding of III2, III3, and III11 is important for superFN aggregation in full-length FN.

We also tested the full-length FN disulfide mutants in cell culture to see whether they could form a matrix. The disulfide bonds in III2 (FN2SS and FN2–3–11SS) decreased the FN matrix by ∼20% relative wild-type FN and FN3–11SS (Fig. 9, B and C, and supplemental Fig. S7). The ability of III2 to unfold appears to be a factor but not indispensable for FN matrix assembly.

**DISCUSSION**

The importance of FNIII domain unfolding was originally pointed out by Litvinovich et al. (48), who discovered that individually expressed III2 was very unstable and easily formed an amyloid-like aggregate. Another example was provided by recent reports that III3 spontaneously unfolds and binds anastellin, a mechanism that seems important for initiating or forming superFN aggregation (24, 37, 49). In the present study, we discovered that the instability of III2 was also important for superFN-like aggregation of the small fragment III1–2. Moreover, the disulfide mutations in full-length FN verified that the instability of FNIII domains plays a role in superFN aggregation. In the case of FN matrix formation, the disulfide lock in III2 caused a 20% reduction of matrix, whereas the locks in III3 and III11 had no effect. This is consistent with the previous demonstration that FN lacking III2 was still able to assemble the matrix, although it was reduced somewhat (26). These findings suggest that the interaction between I1–9 and III2 is not essential for matrix assembly but is one of several redundant binding interactions between FN molecules. The I1–5 deletion (20) or I1–9 deletion3 of full-length FN eliminates its ability to form the matrix, suggesting that I1–9 has more than one assembly site and may be involved in all FN-FN interactions. It is also possible that I1–9 plays another role in assembly such as binding to a cell surface receptor (possibly an integrin) (9, 50–52).

The III1–2KADA mutant was first designed and characterized by Vakonakis et al. (32). They reported that wild-type III1–2 eluted from a gel filtration column ahead of a 29-kDa standard, whereas the KADA mutant migrated in the void volume, ahead of a 150-kDa standard. We determined an Rs of 2.5 nm for both the wild type and KADA mutant, and both proteins sedimented identically at 1.9S (supplemental Fig. S4). As a quality check, we used a simplified Siegel-Monte calculation (47), M = 4,205 Rs, to determine a mass of 20 kDa, which is close to the actual mass of the protein, ∼24 kDa. Our FRET constructs also did not show any significant difference between the wild type and KADA mutant, and both proteins sedimented identically at 1.9S (supplemental Fig. S4). As a quality check, we used a simplified Siegel-Monte calculation (47), M = 4,205 Rs, to determine a mass of 20 kDa, which is close to the actual mass of the protein, ∼24 kDa. Our FRET constructs also did not show any significant difference between the wild type and KADA mutant. We thus found no evidence for a conformational change induced by the KADA mutations. Vakonakis et al. (32) also reported that the KADA mutant, but not the wild type, had a high affinity for I1–5. We found no binding to the larger I1–9 by the gelatin precipitation assay or by FRET analysis.

The results of Karuri et al. (33), who studied the conformation of III1–2 by FRET, add additional contradictions. In their

3 T. Ohashi and H. P. Erickson, unpublished observation.
study, the KADA mutations enhanced the FRET signal, suggesting that the KADA mutant has a more compact conformation than that of wild type. This is the opposite of the Vakonakis model (32). However, their KADA mutant appeared to bind 1′–9, as shown by a reduction in the FRET signal. We found no change in FRET with 1′–9 and therefore no indication of binding. Three laboratories used slightly different constructs and methods to analyze the III1–2KADA mutants, so they may not be directly comparable. The apparent contradictions are not subtle, but we have not been able to suggest a resolution. In our hands the KADA mutations have no effect on conformation or activity.

FNI domains are known to interact with intrinsically unstructured FN-binding proteins (FNBP, adhesins) on bacterial cell walls (43, 53, 54). The NMR structure of a complex between 1′–2 and FNBP from Streptococcus dysgalactiae showed that the unstructured FNBP formed additional tandem β strands on the FNI domains, a structure designated as a “tandem β-zipper” (53). The crystal structures of FNBP from Staphylococcus aureus also showed a tandem β strand arrangement with FNI domains (54). A similar structure containing additional β strands was reported recently in a complex of 8′–9 and type I collagen α1 chain (55). We found that domain III2, destabilized by deletion of the G strand, bound to 1′–9, and the binding was completely inhibited by a bacterial adhesin (FUD) (Table 1). Although there are no direct structural data for the FUD–1′–9 complex, circular dichroism and modeling suggested that the FUD bound by β strand addition (44). Therefore, we speculate that the binding of unfolded III2 to 1′–9 may use a tandem β-zipper mechanism similar to known adhesins. The binding to 1′–9 appeared to be stronger than to the shorter 1′–5 or 6′–9. The FNBP of S. pyogenes also bound more tightly to 1′–9 than to 1′–5 (43, 44, 56). Because 1′–9 is stable against proteolysis in comparison with 1′–9 (see “Experimental Materials” for details), the longer 1′–9 may form a compact conformation that masks a protease-sensitive site. This model is also supported by the hairpin structure of the 6′–9 fragment (57). Structural studies of FN-FNBP complexes indicate that ~10 amino acids are required to form a β strand in a complex with one FNI domain (54, 55). We did not find any obvious sequence similarity between III2 and adhesins. III2 is composed of 93 amino acids (III2ΔG is 82 amino acids) and theoretically could bind nine FNI domains, if fully unfolded. We do not know the structure of the III2 domain in the III1–2/αanastellin aggregate, but the observation that it can bind 1′–9 suggests that some stretch of unfolded III2 peptide may be available to form tandem β-zippers with the FNI domains. Multiple β-zippers might be made with either a compact conformation of 1′–9 (Fig. 10A) or an extended conformation (Fig. 10B).

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