Implications for Collagen Binding from the Crystallographic Structure of Fibronectin $^{6}$FnI$^{1-2}$FnII$^{7}$FnI$^{8,9}$

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Abstract—Collagen and fibronectin (FN) are two abundant and essential components of the vertebrate extracellular matrix; they interact directly with cellular receptors and affect cell adhesion and migration. Past studies identified a FN fragment comprising six modules, $^{6}$FnI$^{1-2}$FnII$^{7-9}$FnI, and termed the gelatin binding domain (GBD) as responsible for collagen interaction. Recently, we showed that the GBD binds tightly to a specific site within type I collagen and determined the structure of domains $^{8-9}$FnI in complex with a peptide from that site. Here, we present the crystallographic structure of domains $^{6}$FnI$^{1-2}$FnII$^{7}$FnI, which form a compact, globular unit through interdomain interactions. Analysis of NMR titrations with single-stranded collagen peptides reveals a dominant collagen interaction surface on domains $^{2}$FnII and $^{7}$FnI; a similar surface appears involved in interactions with triple-helical peptides. Models of the complete GBD, based on the new structure and the $^{8-9}$FnI complex show a continuous putative collagen binding surface. We explore the implications of this model using long collagen peptides and discuss our findings in the context of FN interactions with collagen fibrils.

Collagen fibrils are the basis of vertebrate tissue, and their formation is vital for cell differentiation, cell migration, and embryonic development. The most abundant form, collagen type I, consists of two chains of $\alpha_1$ and one of $\alpha_2$ that intertwine to create right-handed triple helices, which then assemble to make microfibrils and fibers. Fibril formation of type I collagen in vivo requires integrin receptors and fibronectin (FN), a large glycoprotein composed of three domain classes, FN(I), FN(II), and FN(III) (4, 5). FN forms fibrils in the extracellular matrix and interacts with denatured collagen (gelatin) or isolated collagen chains. The interaction site on FN is the gelatin binding domain (GBD), consisting of four FnI and two FnII modules ($^{6}$FnI$^{1-2}$FnII$^{7-9}$FnI) (6). The first structure of a FN-collagen complex, a $^{8-9}$FnI fragment together with a single-stranded collagen peptide was recently determined (7). This structure and subsequent biophysical analysis suggested that $^{8-9}$FnI preferentially binds to unwound collagen. Interestingly, the proposed binding site for FN on collagen I coincides with the cleavage site for metalloproteinase 1, the so-called collagenase site (8–10). This site is hydrophobic and relatively low in proline and hydroxyproline residues, rendering the triple helix unstable at physiological temperature (11). Although these results offered some answers on FN-collagen binding, the role of the remaining four domains in GBD was unclear. The solution NMR structure of $^{6}$FnI$^{1-2}$FnII (12) suggested that $^{1}$FnII reorients flexibly with respect to $^{6}$FnI$^{1-2}$FnII. However, $^{6}$FnI$^{1-2}$FnII shows substantially decreased binding to gelatin (13) and collagen peptides (14) compared with $^{6}$FnI$^{1-2}$FnII$^{7}$FnI, implying a significant role for the $^{7}$FnI domain in this interaction. Here, we present the crystallographic structure of the $^{6}$FnI$^{1-2}$FnII$^{7}$FnI fragment at a resolution of 3.0 Å. We compare this new structure with the previous solution structure of $^{6}$FnI$^{1-2}$FnII and show that $^{1}$FnII and $^{2}$FnII jointly form an interface for $^{7}$FnI; the presence of this interface in solution is supported by analysis of the NMR chemical shifts of these fragments. NMR titrations with single-stranded as well as triple-helical peptides from the collagenase site show that a unique binding surface, involving domains $^{2}$FnII and $^{7}$FnI, is important for collagen binding. Together with previously published data (7), we now offer a model for the complete GBD which suggests that the two GBD subfragments bind collagen in a concerted fashion.

Experimental Procedures

Protein Expression, Purification, and Crystallization—A gene fragment, encoding FN residues 305–515, corresponding to domains $^{6}$FnI$^{1-2}$FnII, and bearing a single amino acid substitution (H307D) and a C-terminal His$_6$ tag (GTKHHH-HHHH) was integrated in Pichia pastoris in a manner analogous to that previously described (15). The H307D substitution does not affect the binding of $^{6}$FnI$^{1-2}$FnII$^{7}$FnI to gelatin-Sepharose columns (data not shown) but increases the solubility of this fragment under physiological pH conditions significantly.
were grown under high density fermentation conditions, using a minimal phosphate medium at pH 3.25 and 30 °C; protein expression was induced by the addition of methanol over the course of 5–7 days. 6FnI1–2FnII7FnI was purified from the expression media by metal affinity chromatography, and the N-linked glycan at Asn430 was truncated by endoglycosidase H treatment, leaving a single N-acetylgalactosamine at this site. The protein was further purified by reverse phase HPLC, lyophilized, and finally run on a Sephadex G-75 size exclusion column equilibrated in a 100 mM NaCl, 10 mM HEPES, pH 7.2, buffer. 6FnI1–2FnII7FnI was concentrated in this buffer to ~25 mg/ml.

Crystallization drops were formed by 1:1 mixtures of protein in the final purification buffer at 17.5 mg/ml concentration, and a 0.2 M (NH₄)₂H₂PO₄, 0.1 M Tris-Cl, pH 8.5, 50% v/v 2-methyl-2-pentanediol solution. Sitting-drop vapor diffusion was employed in 96-well plates, with drop volumes varying between 200 and 400 nl. 6FnI1–2FnII7FnI crystals of lens-like appearance and ~200 μm in the longest axis developed after 6–8 weeks at 20 °C. Crystals were cryoprotected by brief immersion in crystallization mother liquor and frozen in a nitrogen cryostream at 100 K.

X-ray Data Collection and Processing, Structure Determination—Data were collected under cryogenic conditions at the PXIII macromolecular crystallography beamline at the Swiss Light Source (Villigen, Switzerland). Reflection data were indexed by LABELIT (16), refined and integrated in XDS (17), and merged by SCALA (18). The Laue group and space group were suggested by POINTLESS (18) from the unmerged data, and data quality was assessed by PHENIX.xtriage (19) (supplemental Table 1).

Initial structure determination was performed by molecular replacement using PHASER (20) and superimposed ensembles of Fn- and FnII-type modules of known crystallographic structures. Iterative cycles of model building in COOT (21) and refinement using PHENIX.refine (19) with TLS restraints (one chain) resulted in a final model with satisfactory refinement using PHENIX.refine (19) with TLS restraints (one chain) equilibrated in a 100 mM NaCl, 10 mM HEPES, pH 7.2. Structure of 6FnI1–2FnII7FnI was cryoprotected by brief immersion in crystallization mother liquor and frozen in a nitrogen cryostream at 100 K.

RESULTS

Structure of 6FnI1–2FnII7FnI—A FN fragment spanning domains 6FnI1–2FnII7FnI crystallized using the sitting-drop vapor diffusion method as described under "Experimental Procedures." Lens-like birefringent objects grew slowly out of initial light precipitate over the course of ~2 months at 20 °C. Despite the lack of well defined edges, these objects were crystalline in nature and diffracted to ~3.5 Å resolution in synchrotron x-ray sources. Screening around the initial crystallization conditions or use of additives did not improve the morphology of these crystals; however, it was possible to collect a complete 3.0 Å dataset (supplemental Table 1) by screening different crystals from the original conditions. The Matthews coefficient strongly suggested a single 6FnI1–2FnII7FnI molecule/asymmetric unit, and the structure was solved by molecular replacement using two copies of Fn- and FnII-type structural ensembles for a total of four search objects. The initial molecular replacement map was of sufficient quality to allow tracing of interdomain linkers and to establish domain identity and connectivity.

All individual 6FnI1–2FnII7FnI domains adopt canonical structures (12, 29), a result anticipated based on chemical shift index analysis (30) of this fragment in solution. Fn-type domains form a β-sandwich with antiparallel two-stranded and three-stranded β-sheets. FnII-type domains are characterized by extensive loop segments and only two short antiparallel two-stranded sheets. Both FnI- and FnII-type domains feature two disulfide bridges that contribute substantially to these domain folds.

α₁(II) Gly778–Arg816 peptide was purchased from GL Biochem (Shanghai) as HPLC-purified, lyophilized powder. Protein concentration was established by UV absorbance at 280 nm, and peptide concentration was initially estimated from dry weight. Protein solutions were dialyzed overnight against 150 mM NaCl, 20 mM Na₂HPO₄, pH 7.2. The pH of peptide solutions was adjusted with 1 M NaOH to match this buffer. ITC experiments (VP-ITC; MicroCal) were performed as follows: one injection of 2 µl followed by 44 injections of 5 µl at 0.5 µl/s. The stirring speed was 307 rpm; the delay between the injections was 210 s. To take into account heats of dilution, blank titrations were performed by injecting peptide solution into buffer, and the averaged heat of dilution was subtracted from the main experiment. Raw data were processed and fitted to a one-site model using MicroCal Origin software.

Data Deposition—Amino acid composition and numbering for FN fragments correspond to UniProt entry B7ZLF0. α₁(II) and α₁(II) numbering (accession numbers P02452 and P02458, respectively) begins at the estimated start of the helical region. O in peptide sequences denotes 4-hydroxyproline. Structural analysis was performed, and figures were prepared using PyMOL (27). Interdomain interactions were analyzed with the PISA service from the European Bioinformatics Institute (28). Structural data have been deposited in the Protein Data Bank under accession number 3MQL, and NMR chemical shift assignments are available in the BioMagResBank under accession number 16841.

Structure of the 6FnI1–2FnII7FnI Fibronectin Fragment
In contrast to common beads-on-string models of multidomain proteins (31), the crystallographic structure of 6FnI1–2FnII7FnI adopts a pyramidal shape. As shown in Fig. 1A, 6FnI1–2FnII forms an approximately equilateral triangle with sides of ~35 Å. From this base, 7FnI projects out by ~38 Å (Fig. 1B) and forms extensive hydrogen-bonding interactions with a 7FnI domain from a crystallographic 2-fold symmetry-related molecule. Other crystal contacts include further β-sheet extensions through 7FnI-7FnI and 6FnI-6FnI interactions; however, it should be noted that similar extensions are common among crystallographic structures of FnI-type domains (29, 32). No evidence of protein oligomerization was apparent in solution NMR experiments even at sample concentrations of >1 mM (data not shown).

The compact 6FnI1–2FnII7FnI conformation is maintained through interdomain interactions that show a remarkable degree of conservation (supplemental Fig. 1). 6FnI interacts with 2FnII in a manner essentially identical to that observed in the solution 6FnI1–2FnII structure (12), burying ~395 Å² of solvent-accessible surface area (Fig. 2A). The Cα root mean square deviation for 6FnI-2FnII between the solution structure and our crystallographic model is only 1.7 Å, whereas the individual domains differ by 1.0 and 1.1 Å for 6FnI and 2FnII, respectively. Residues 314–323 of 6FnI, and 414–421 and 448–449 of 2FnII are primarily involved in forming the interface, with significant contributions from Met320, Ser415, Ala418, Leu419, Thr448, and Thr449 (Fig. 2A).

The solution structure of 6FnI1–2FnII featured a well defined 1FnII domain which was, however, mobile in relation to the 6FnI-2FnII complex (12). In contrast, our crystallographic model shows the formation of a 1FnII-2FnII interface (Fig. 2B) burying ~330 Å². 1FnII rotates and translates toward 2FnII and 7FnI (Fig. 3, A and B, and below); this motion places 1FnII outside the ensemble of conformations shown in the solution structure of 6FnI1–2FnII. The 1FnII-2FnII interface involves primarily Tyr312, Val408, and Pro462 as well long range hydrogen bonds between Val315 O'-Asn416 N02, Gln321 O'-Leu419 N, and Tyr316 O'-Asn416 O'. In addition, 1FnII helps to structure the relatively long linker (residues 461–468) connecting 2FnII and 7FnI, which in turn stabilizes the 7FnI conformation relative to the remaining domains. 7FnI interacts with both 1FnII and 2FnII across an interface burying ~390 Å² of solvent-accessible surface area (Fig. 2C). Residues Thr365, Ser390, Asn391, Met463, Ala464, His466, Ile480, and Gly502 are involved in hydrophobic burial and hydrogen bonding interactions, whereas Arg479 and Ile480 help anchor 7FnI to the structured 2FnII-7FnI linker (Fig. 2C).

Crystallographic versus Solution 6FnI1–2FnII7FnI Conformation—To evaluate whether the 6FnI1–2FnII7FnI conformation observed is present in solution, we compared the NMR chemical shifts of 6FnI1–2FnII with those of 6FnI1–2FnII7FnI under the same experimental conditions. Addition of 7FnI to the fragment causes chemical shift differences that extend further than the immediate attachment point (i.e. the C terminus of 2FnII; see supplemental Fig. 2). Fig. 3, C and D, shows residues whose NMR resonances differ by more than 1
S.D. compared with the average; these include: residues at the 6FnI–2FnII interdomain linkers (Ala346, Thr348, Gln349, Thr402, Asp403, and Leu407); residues at the new 1FnII-2FnII interface (Tyr372, Cys334) and numerous residues on both 1FnII and 2FnII that circumscribe the 7FnI anchoring point (Fig. 3D). These differences correlate well with differences between the solution 6FnI1–2FnII structure and our model and are likely to be in the microsecond range based on spectral properties. However, the lack of sequential assignments and a structural model for 6FnI1–2FnII7FnI prevented further analysis of these data at the time.

As shown in supplemental Fig. 2, chemical shift perturbations larger than 2 S.D. compared with the average (red bars) localize exclusively on residues of 7FnI7FnI, and smaller but significant perturbations (1–2 S.D., yellow bars) largely follow the same pattern. Mapping these perturbations on the 6FnI1–2FnII7FnI structure shows a dominant interaction surface (Fig. 4) spanning domains 2FnII7FnI and primarily involving residues Asp427, Tyr452, Phe458, Gly500, Arg501, Gly504, Trp506, and Thr507. Although 1FnII does not participate in this interaction interface it stabilizes the 7FnI orientation thereby contributing to the formation of a continuous 2FnII7FnI binding surface.

Our previous study of collagen peptides binding to GBD fragments (7) and work since then have identified αI(I) residues Gly778–Gly799 as the single-stranded peptide with the highest known affinity for 6FnI1–2FnII7FnI in type I collagen. This peptide is adjacent to the matrix metalloproteinase 1 cleavage site and coincides with the collagen fragment implicated in FN binding using fluorescent probes (6) as well as competition assays between serum FN and collagen (34). Several other peptides are known to bind with low affinity, with Kd values of well over 1 mM (7), but these tend to cause few chemical shift perturbations. Analysis of these weak interactions showed effects primarily on Trp385 (1FnII), Trp445 (2FnII), and residues in their vicinity. These residues belong to a hydrophobic pocket on the surface of FnII-type modules that is known to interact weakly with collagen-like peptides (14, 35); in our crystallographic model the same sites are occupied by two 2-methyl-2,4-pentanediol molecules from the crystallization solution. We believe that these lower affinity interactions correspond to non-specific binding events, possibly related to the generic gelatin affinity displayed by many FnII-type modules (36–38). In contrast, our higher affinity αI(I) Gly778–Gly799 peptide interacts with a unique interface involving only one of these two hydrophobic pockets; the peptide is likely to be a specific ligand.
bound collagen peptide (7) is co-linear with the collagen binding interface in 6FnI–2FnI–7FnI, indicating the presence of a single continuous binding surface. 8–9FnI binds collagen in an antiparallel orientation; the last collagen residue interacting with 8–9FnI is Arg972 of α(I), which suggests that residues C-terminal to Arg972 would bind 4FnI–2FnII–7FnI. However, isolated peptides spanning α(I) residues Gly796–Arg816 interact with 6FnI–2FnII–7FnI only very weakly as judged by NMR (7).

To test whether 6FnI–2FnII–7FnI and 8–9FnI can bind collagen in a concerted fashion in the context of the GBD, we performed ITC experiments using GBD, 8–9FnI, and a long peptide, α(I) residues Gly778–Arg816. This peptide includes the high affinity 8–9FnI epitope and extends to potentially cover the 6FnI–2FnII–7FnI collagen binding surface observed here (Fig. 4). Although this peptide was not designed to form triple helices, a degree of helical structure was detected by CD at conditions similar to those used for ITC (supplemental Fig. 4). The partial helical structure of α(I) Gly778–Arg816 may impede 8–9FnI binding as it will necessitate peptide unwinding and adoption of an extended conformation prior to interaction (7). As seen in supplemental Fig. 6, 8–9FnI binds α(I) Gly778–Arg816 with relatively weak affinity ($K_d \approx 115 \mu M$). However, under the same conditions, GBD bound α(I) Gly778–Arg816 $\approx 3$ times more tightly. Although the increase in affinity is moderate, it is in agreement with our model for concerted binding of the two GBD subfragments to collagen.

**DISCUSSION**

8–9FnI was previously shown to interact tightly ($K_d \approx 5 \mu M$) with a specific collagen peptide derived from the α(I) chain (7). Crystallographic analysis of this complex also allowed us to identify a number of putative sites for 8–9FnI on that chain. The role of 6FnI–2FnII–7FnI in the GBD, however, remained unknown. Here, we presented the crystal structure of 6FnI–2FnII–7FnI, which together with the structure of 8–9FnI (7) and extensive NMR titration analysis allowed us to model the structure of the full GBD of FN. Our results offer new insights into how FN may interact with collagen.

GBD adopts an elongated structure in our model in which the two spatially distinct subfragments show good binding to the same collagen type I sequence, residues Gly788–Gly795. A question thus emerges as to how both 6FnI–2FnII–7FnI and 8–9FnI can associate with the same epitope. Previous hypotheses included GBD binding to two of the three strands in a single triple helix, or GBD associating with multiple strands across the collagen microfibril (7). However, neither of these hypotheses is plausible as they would necessitate collagen chain displacement beyond the local fluctuations believed to be present under physiological conditions (40).

An alternative hypothesis emerges from our GBD model and the ITC data: a continuous collagen binding site on GBD can interact with a single long collagen epitope in a concerted fashion. Although the available evidence is tentative, the interplay between the two GBD subfragments in binding collagen chains could facilitate tight attachment. It is intriguing to speculate on the relative contributions of the GBD subfragments to such a binding event. 6FnI–2FnII–7FnI was shown here, and earlier (7), to form relatively weak, transient complexes with single-
stranded and triple-helical forms of collagen peptides; in contrast 8–9FnI binds more tightly to unwound collagen strands. There is a growing body of evidence that weak, transient encounter complexes precede formation of tight intermolecular complexes in biological systems (41, 42). Thus, it is possible that 6FnI1–2FnII7FnI binds adjacent to the collagenase site and lar complexes in biological systems (41, 42). Thus, it is possible with domains 7FnI and 9FnI. Experiments with fluorescently labeled collagen peptides showed that Zn$^{2+}$ at concentrations of hundreds of micromolar interferes with collagen binding by the GBD. These concentrations are much higher than the physiological levels of Zn$^{2+}$ in blood, 10–15 μM (47–49), although Zn$^{2+}$ levels may be higher locally in specific tissues. Thus, Zn$^{2+}$ could play a regulatory role in the FN-collagen interaction through conformational changes; in this case the GBD model we propose here would correspond to the collagen binding form of FN.

The role of FN-collagen binding in vivo has been assessed over many years; putative FN roles include scavenging of collagen fragments (50–52), stabilization and protection of collagen fibrils (4), and acting as a molecular tag for collagen proteolysis. FN has also been reported to be an opsonic protein (53, 54), enhancing phagocytosis when bound to a target. Numerous examples of FN localization to damaged tissue are known, including localization to burned skin as well as injured liver (50, 55–57). Given the ubiquitous distribution of FN in the extracellular matrix as well as in plasma, we believe that the FN-collagen interaction may serve more than one purpose. Considering the widespread interest in extracellular matrix components and their interactions in the field of biomaterials and prosthetics (58, 59), our structural data may have significant influence on future biomedical as well as medical studies.

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**Structure of the $^6$FnI$^{1-2}$FnI$^7$FnI Fibronectin Fragment**

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