Extra-domain B in Oncofetal Fibronectin Structurally Promotes Fibrillar Head-to-tail Dimerization of Extracellular Matrix Protein

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Background: Extracellular matrix and plasma forms of fibronectin show fibrillar and compact conformations, respectively. X-ray analysis of the FnIII7B89 fragment reveals a rotation between domains 7 and 8 upon insertion of B and, unexpectedly, homodimerization. Together with alternating disulfide bridges at the C-terminal tail, the homodimerization suggests a model for macromolecular fibril formation.

Results: X-ray analysis of the FnIII7B89 fragment reveals a rotation between domains 7 and 8 upon insertion of B and, unexpectedly, homodimerization. ED-B in oncofetal Fn appears to stabilize oligomerization.

Conclusion: ED-B in oncofetal Fn appears to stabilize oligomerization. Together with alternating disulfide bridges at the C-terminal tail, the homodimerization suggests a model for macromolecular fibril formation.

The type III extra-domain B (ED-B) is specifically spliced into fibronectin (Fn) during embryogenesis and neoangiogenesis, including many cancers. The X-ray structure of the recombinant four-domain fragment FnIII7B89 reveals a tightly associated, extended head-to-tail dimer, which is stabilized via pair-wise shape and charge complementarity. A tendency toward ED-B-dependent dimer formation in solution was supported by size exclusion chromatography and analytical ultracentrifugation. When amending the model with the known three-dimensional structure of the FnIII10 domain, its RGD loop as well as the adhesion synergy region in FnIII9–10 become displayed on the same face of the dimer; this should allow simultaneous binding of at least two integrins and, thus, receptor clustering on the cell surface and intracellular signaling. Insertion of ED-B appears to stabilize overall head-to-tail dimerization of two separate Fn chains, which, together with alternating homodimer formation via disulfide bridges at the C-terminal Fn tail, should lead to the known macromolecular fibril formation.

In loose connective tissue, Fn is secreted from fibroblasts as a disulfide-linked tail-to-tail dimer and deposited locally in the ECM. There, it interacts with its predominant cellular receptor integrin, with other matrix proteins such as collagen and fibrin, and also with the proangiogenic VEGF. Upon binding to its receptors, the covalently dimerized Fn gets accumulated on the cell surface, where it multimerizes into insoluble fibrils. The resulting multifunctional matrix seems to bring together various ECM components and cell surface receptors. Apart from such tissue Fn, there is an abundant fraction of soluble plasma Fn circulating in the bloodstream, which assumes a non-functional, compact conformation but can be deposited at the ECM upon reactivation.

Cell surface Fn consists of two mostly identical polypeptide chains, each with a mass of ~250 kDa, which are joined by a pair of disulfide bridges at their C termini. Each monomer comprises an ordered domain organization with 30–32 small modules that are arranged in a repetitive manner like beads on a string. There are three different types of repeating modules in Fn, which are all dominated by a sandwich of antiparallel β-sheets. Type I domains (FnI), which are smaller in size, are found at the N and C termini and have been shown to be critical for matrix assembly of Fn molecules. Only two domains of Fn belong to the type II fold (FnII) and are found near the N terminus, where they are surrounded by type I domains. The most abundant type of domains is classified as type III (FnIII) and constitutes the larger internal part of Fn, including the 15–17 repeats referred to as the cell-binding segment. Unlike the type I and II modules, the type III fold is not stabilized by intradomain disulfide bonds.

The cell-binding FnIII segment forms the most heterogeneous part of this ECM protein as there are ~20 different variants described for humans that arise from alternative splicing events at three sites, especially in the variable stretch, which lacks homology to the FnI, FnII, or FnIII domains and is located between FnIII repeats 14 and 15 (13, 14). Expression of these isoforms is tissue- and cell-specific, hence suggesting functional significance. Importantly, initiation of angiogenesis is an
essential event during tumorigenesis and often accompanied by increasing expression levels of two so-called “oncofetal” Fn isoforms, which are otherwise confined to embryonic development and wound healing (15).

These oncofetal Fn variants incorporate additional type III domains dubbed extra-domain A (ED-A) (16), inserted between FnIII11 and FnIII12, and extra-domain B (ED-B) (17), which is inserted between FnIII7 and FnIII8. ED-B especially has been shown to be spliced into Fn only during embryogenesis and neoangiogenesis such as occurring in cancer or other pathologies, but is undetectable in healthy mature blood vessels. Consequently, ED-B has attracted clinical attention as a diagnostic marker to distinguish tumor vessels from normal vasculature (18). In fact, ED-B-positive Fn has been detected in 62 of 165 primary tumors of various histotypes (19) as well as in neovascular structures (20). Nevertheless, the physiological function of oncofetal Fn in angiogenic processes is still obscure.

The splicing sites of ED-A and ED-B are both close to the cell binding RGD(5) motif and the so-called adhesion synergy region (21, 22), which are located in FnIII10 and FnIII9, respectively, and constitute ligands of the integrins α5β1 (23, 24). Mutations in these motifs or inhibition by RGD-containing peptides lead to a reduction in Fn matrix assembly (25–27), confirming the importance of these molecular interactions for the formation of extracellular Fn fibrils. Hence, it was speculated that insertion of ED-A or ED-B might trigger a (global and/or local) conformational change in Fn, which in turn could promote multiple integrin binding events (28–32).

In the present study, we have performed an x-ray structural analysis of ED-B in the context of its neighboring domains FnIII7, FnIII8, and FnIII9, revealing a previously unseen mode of homodimerization by this internal segment of Fn and, thus, suggesting a specific structural role of ED-B in the supramolecular organization of intercellular Fn.

**EXPERIMENTAL PROCEDURES**

**Vector Construction**—The expression plasmids pASK75-Fn7B89-His6, pASK75-Fn7B8-His6, and pASK75-Fn7–10-His6 were derivatives of pASK75 (33) encoding various Fn fragments fused to a C-terminal His6 tag. Gene fragments for Fn domains 7, 8, 9, and 10 were amplified from the plasmid pET11b-FN7–10-FN (34) using *Pfu Ultra* II Fusion HS DNA Polymerase (Agilent, Santa Clara, CA) and the following primer pairs: 5′-AGT GTC CAT CAT ATG CCA TTG TCT CCA CC-3′ and 5′-CGA CTG TCT AGA CAG TCG ACG CCT GTA AGA CCC TAC TAC CAT ATG CAG TCT AGA TCT ACT GTA TCC CCT CCA CC-3′; and 5′-AGT GTA TTC CAT ATG CCA TTG TCT CCA CC-3′ and 5′-CGA CTG TCT AGA CAG TCG ACG CCT GTA AGA CCC TAC TAC CAT ATG CAG TCT AGA TCT ACT GTA TCC CCT CCA CC-3′ for domain 7; 5′-CAG CAG ACC GCC GTT CCT CCT CCC AC-3′ and 5′-CGA TTG ACA ACT AAC AAC CAG GTA TTA G-3′ for the domain tandem 8–9 (restriction sites for Ndel, BglII, and SacII are underlined; the last primer generated a blunt end). The 273-bp fragment encoding ED-B (17) was obtained by gene synthesis via PCR amplification using primers 5′-GGA TGA CAA GGA AAG TGT CC-3′ and 5′-GCT ACC TGG TGG GCC GGC GTC TGC TGG G-3′. All PCR products were digested with the corresponding restriction enzymes, purified by agarose gel electrophoresis, and successively cloned on a derivative of pASK75-His6, which provides for the unique Ndel and Eco47II (blunt end) restriction sites at the start of the reading frame and in front of the His6 tag, respectively, resulting in pASK75-Fn7B89-His6, pASK75-Fn7B8-His6, and pASK75-Fn7–10-His6, which was generated by insertion of the gene segment encoding domain 10, amplified with primers 5′-GAT GTT CCG AGG GAC CTG-3′ and 5′-GCT TGT TGG GTA ATT AAT GGA AAT TGG-3′ from pET11b-FN7–10, via the Eco47II site into pASK75-Fn7B89-His6, which had been constructed in the same way as pASK75-Fn7B89-His6 but with omission of the segment encoding ED-B. All plasmid constructions were confirmed by analytical restriction digest as well as double-stranded dyeoxy sequencing of the Fn gene inserts (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA).

**Expression and Purification of Fn Fragments**—Recombinant FnIII7B89 was produced as soluble protein in the cytoplasm of *Escherichia coli* BL21 (36). A 2-liter shake flask culture of bacteria harboring pASK75-Fn7B89-His6 was grown at 37 °C in LB medium containing 100 μg/ml ampicillin in mid-log phase. After addition of 0.2 mg/liter anhydrotetracycline (Acros, Geel, Belgium), growth was continued for 6 h. Cells were pelleted by centrifugation, resuspended in 30 ml of ice-cold chromatography buffer (1 mM NaCl, 40 mM NaPO4, pH 7.5) and lysed by sonification (S250D cell disruptor, Branson, Danbury, CT). The cleared lysate was dialyzed against 1 mM NaCl, 40 mM NaPO4, pH 7.5, and applied to an IDA-Sepharose column (GE Healthcare) charged with 10 mM ZnSO4 and equilibrated with chromatography buffer. Bound protein was eluted with a concentration gradient of 0 to 250 mM imidazole/Tris in the chromatography buffer (37). Fractions containing the recombinant protein were identified by SDS-PAGE, supplemented with 1 mM EDTA, and dialyzed against 40 mM Hepes/NaOH, pH 7.4. Then, the protein was loaded onto an ion exchange chromatography column (Resource Q, GE Healthcare) equilibrated with 150 mM NaCl, 20 mM Hepes/NaOH, pH 7.4. FnIII7B89 was eluted in a concentration gradient between 150 and 300 mM NaCl, typically yielding 40 mg of purified protein from a 2-liter culture.

Recombinant Fn fragments FnIII7B8 and FnIII7–10 were expressed in *E. coli* from the appropriate plasmids described above in a similar manner using French pressure lysis and affinity purification on a Ni(II)-charged HiTrap column (GE Healthcare), followed by Resource Q chromatography. Purity was checked to be better than 95% via SDS-PAGE, and protein concentrations were measured by UV absorption using calculated extinction coefficients from ProtParam (38).

**Biochemical Characterization of Fn Fragments**—Analytical size exclusion chromatography (SEC) was carried out on a 24-ml bed volume Tricorn column (Superdex 200 10/300 GL; GE Healthcare) at a flow rate of 0.5 ml/min using AKTA purifier instrumentation (GE Healthcare). FnIII7B8, FnIII7B89, and FnIII7–10 were analyzed in three different running buffers of increasing ionic strength, all containing 20 mM Hepes/NaOH,
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pH 7.5: (A) 60 mM NaCl, (B) 140 mM NaCl, (C) 300 mM NaCl, thus increasing the ionic strength from ~80 to 320 mM. For each buffer condition, the column was calibrated (using a semilogarithmic linear plot) with the following set of protein size standards (all from SIGMA, Steinheim, Germany): apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). Cytochrome c did not yield a sharp peak in running buffer A and, thus, was not used under this condition. The void volume was determined with blue dextran. 0.5 mg of each FnIII7B89, FnIII7B89, and FnIII7–10 were equilibrated against the respective running buffer via repeated dilution and concentration by ultra-filtration and finally concentrated to a volume of 150 μl using a 10-kDa cut-off concentrator (Amicon Ultra-4, Millipore, Billerica, MA) before application to the SEC column.

Analytical ultracentrifugation (AUC) was performed in a Beckman Optima XL-1 analytical ultracentrifuge with a Ti-60 rotor equipped with a UV-visible and an interference detection unit (Beckman, Fullerton, CA). Solutions of FnIII7B89 at concentrations of 12 μM in 20 mM Hepes/NaOH, 20 mM NaCl, 1 mM DTT, 0.02% (w/v) NaN3, pH 7.5, were applied to six-sector 12-mm path length cells. The samples were centrifuged at 23,000 rpm and 20 °C for 48 h until equilibrium was reached, whereby the protein gradient was measured by UV absorption at 290 nm. Data analysis was carried out with Kaleidagraph software (Synergy Software, Reading, PA) as described previously (39) using a density of 1.000 g/ml for the solvent and a standard value of 0.74 ml/g for the partial specific volume of the protein.

Protein Crystallization and Structure Determination—Crystals of FnIII7B89 were grown in sitting drops by vapor diffusion after buffer exchange of the purified protein against 20 mM NaCl, 10 mM Hepes/NaOH, pH 7.5, and concentration to 10 mg/ml as described above. Crystals were obtained as bunches of needles or plates in the presence of PEG3350 (SIGMA) as precipitant. Data were collected from the largest plates available, exhibiting a smallest dimension of ~10 μm. These crystals showed poor diffraction on a rotating anode generator but diffracted up to 2.4 Å resolution at a synchrotron x-ray source. Best diffracting crystals were obtained by mixing 0.5 μl of the FnIII7B89 protein solution with 0.5 μl of reservoir containing 26% (v/v) PEG3350, 5% (v/v) isopropanol, 0.2 m NaCl, 0.1 mM Tris/HCl, pH 9.0, attaining dimensions of ~500 × 100 × 10 μm³, within 2 weeks. Cryo-protection was achieved by immersing in reservoir solution supplemented with 20% (v/v) ethylene glycol. The crystals were harvested with MicroMounts (Molecular Dimensions, Suffolk, UK) and flash-cooled in liquid nitrogen. Diffraction data were collected from a single crystal at BESSY beamline 14.1. The data were indexed, integrated, and scaled with the XDS package (40). The crystal belonged to the space group C2, containing one protein molecule per asymmetric unit (Table 1).

The structure was solved by molecular replacement as implemented in PHASER (41), using the Fn domains 7, 8, and 9 from PDB entry 1FNF (34) as search models. Their mutual structural similarity required placing of the Fn domains in a sequential order, starting with the two-domain fragment 8–9, followed by domain 7. After rigid body and restrained refinement using REFMAC (42), the model for ED-B was manually built in iterative cycles with COOT (43) using restrained refinement with REFMAC. Translation/Libration/Screw (TLS) parameters were determined with TLSMD (44) and used for final TLS and restrained refinement with REFMAC (45). The quality of the resulting model was validated with COOT and MolProbity (46). Graphical representations were prepared using PyMOL (47), whereas secondary structure elements were assigned with DSSP (48), and electrostatic surfaces were calculated with APBS (49). Structural superposition was performed with SUPERPOSE (50), interfaces were analyzed with Protein Interfaces, Surfaces and Assemblies (PISA) (51), and radii of gyration were calculated with MOLEMAN2 (52).

**RESULTS**

**X-ray Analysis and Overall Structure of FnIII7B89—The FnIII7B89 fragment (human Fn isoform 1, residues 1173 to 1539; NCBI GenBank™ accession no. NP_997647.1) was expressed as a soluble protein in the cytoplasm of E. coli, purified via a C-terminal His6 tag, and crystallized at pH 9.0 using PEG3350 as precipitant. Crystals of FnIII7B89 typically grew in bunches and showed either needle- or plate-like morphology. X-ray data were collected from the largest plates that could be separated, diffracting up to 2.4 Å resolution at the BESSY synchrotron.**

FnIII7B89 crystallized in the space group C2 with one molecule per asymmetric unit (Table 1). The strongly anisotropic unit cell dimensions reflect the crystal morphology. Although dense packing contacts could be seen along the a- and c-axes, packing along the b-axis appeared very weak (see below). Interpretable electron density was observed for residues 1173–1539, hence covering the entire fragment, including the additional Ala residue that links the C terminus of FnIII7 to the His6 tag.

The crystal structure of FnIII7B89 reveals a chain of four contiguous FnIII domains in an extended conformation (Fig. 1) with approximate dimensions of 150 × 30 × 30 Å³. Each of the domains assumes the classical FnIII fold, comprising a sandwich of two antiparallel β-sheets with three (a-b-e) and four (g-f-c-c') β-strands, respectively. Pair-wise comparison of the four individual domains via matched secondary structures revealed the largest root mean square deviation (r.m.s.d.) between ED-B and FnIII7 with 1.56 Å (for 87 Ca positions) and the smallest r.m.s.d. between FnIII8 and FnIII9 with 0.95 Å (for 88 Ca positions). ED-B is most similar to FnIII8 with an r.m.s.d. of 1.19 Å (for 88 Ca positions). Larger differences in the backbone conformation between the individual domains were mostly found in the CC' and BC loops. FnIII7 revealed considerable structural deviations in its AB and CC' loops, both of which harbor a one-residue insertion compared with the other FnIII domains. The average B values of 53, 82, 85, and 24 Å², respectively, for the individual domains FnIII7, ED-B, FnIII8, and FnIII9 showed significant variation, indicating increasing structural order toward both ends of the tetra-domain fragment of Fn, a somewhat unexpected finding to be discussed further below.

Comparison of FnIII7B89 with Previously Elucidated Fn Structures—Comparison of the individual domains FnIII7, ED-B, FnIII8, and FnIII9 with previously determined structures
of ED-B alone (53) (PDB code 2FNB), the two-domain fragment Fn\textsuperscript{III}B8 (31) (PDB code 2GEE), and a tetra-domain fragment lacking ED-B, Fn\textsuperscript{III}7–10 (34) (PDB code 1FNF), revealed elevated r.m.s.d. values for the domains Fn\textsuperscript{III}7 and, in particular, ED-B. Notably, ED-B in the presently described structure of Fn\textsuperscript{III}7B89 showed r.m.s.d. values of 1.60 and 1.94 Å (for 89 C\textsubscript{α} positions), respectively, when superimposed with the NMR structure of ED-B (model 1) (53) or with the x-ray structure of FnIIIB8 (31). Nevertheless, the mutual r.m.s.d. between the later two structures was even larger with 2.42 Å. In fact, ED-B in context of its neighboring domains Fn\textsuperscript{III}7 and Fn\textsuperscript{III}8 more closely resembles the structure of the isolated domain solved by NMR spectroscopy than that of the recently published Fn\textsuperscript{III}B8 crystal structure, which lacks Fn\textsuperscript{III}7 on the N-terminal side.

In contrast, domain Fn\textsuperscript{III}8 as part of Fn\textsuperscript{III}7B89 had r.m.s.d. values of only 0.84 Å (for 90 C\textsubscript{α} positions) and 0.58 Å (for 91 C\textsubscript{α} positions) if compared with the two-domain fragment Fn\textsuperscript{III}B8 (31) or the tetra-domain fragment Fn\textsuperscript{III}7–10 (34), respectively. On the other hand, the r.m.s.d. between Fn\textsuperscript{III}7 as part of Fn\textsuperscript{III}7B89 and as part of Fn\textsuperscript{III}7–10 was higher with 1.68 Å (for 93 C\textsubscript{α} positions), which is largely due to conformational variations in the CC and AB loops. Domain Fn\textsuperscript{III}9 of Fn\textsuperscript{III}7B89 showed an r.m.s.d. of 1.25 Å (for 91 C\textsubscript{α} positions) if compared with Fn\textsuperscript{III}7–10, which again is the result of a large conformational deviation in the CC loop, with a local backbone r.m.s.d. of up to 7.67 Å (Fig. 1C).

At first glance, comparison between the Fn\textsuperscript{III}7B89 crystal structure solved here with those of FnIIIB8 and Fn\textsuperscript{III}7–10 suggested a similar overall extended conformation. However, a more detailed inspection of the domain boundaries revealed unexpected structural differences, especially in the linker regions connecting domains Fn\textsuperscript{III}7, ED-B, and Fn\textsuperscript{III}8. In fact, insertion of ED-B into Fn\textsuperscript{III}7B89 and the resulting connections Fn\textsuperscript{III}7–ED-B and ED-B–Fn\textsuperscript{III}8 leads to significantly altered rotational and tilt angles along the Fn chain compared with the directly linked Fn\textsuperscript{III}7–Fn\textsuperscript{III}8 moiety in Fn\textsuperscript{III}7–10. Interestingly, the AB loop of Fn\textsuperscript{III}7 seems to restrain the tilt angle between domains Fn\textsuperscript{III}7 and ED-B (a segment not structurally investi-

| Data collection and refinement statistics |
|-----------------------------------------|
| **Data collection**                     |
| Space group                             | C2                                      |
| Unit cell parameters                    | a = 211.98 Å, b = 29.12 Å, and c = 82.24 Å; |
| Wavelength (Å)                          | 0.9184                                  |
| Resolution (Å)                          | 30–2.4 Å (2.5–2.4)\textsuperscript{a}   |
| Completeness (%)                        | 99.9 (99.7)                             |
| Unique reflections                      | 18,943 (2147)                           |
| Multiplicity                            | 3.7 (3.7)                               |
| Mean I/σ(I)                             | 18.8 (3.5)                              |
| R<sub>max</sub> (%)                     | 6.6 (49.7)                              |
| Wilson B-factor (Å²)                    | 44.1                                    |
| **Refinement**                          |
| Resolution (Å)                          | 30.0–2.40 (2.46–2.40)                   |
| Reflections (working)                   | 17,966 (1299)                           |
| Reflections (test)                      | 976 (72)                                |
| R<sub>free</sub> (%)                    | 22.8 (29.0)                             |
| R<sub>free</sub> (%)\textsuperscript{a} | 27.7 (36.5)                             |
| Number of protein atoms/waters          | 2822/118                                |
| B-values of protein atoms/waters (Å²)   | 60.4/40.4                               |
| Ramachandran plot                       | 96.2/0.6                                |
| Favorable/outliers (%)                  | 0.009/1.15                              |

\textsuperscript{a} Values in parentheses represent the highest resolution shell.  
\textsuperscript{b} R<sub>free</sub> is calculated as for R<sub>cryst</sub>, but with 5% of the reflections excluded from the refinement.

**FIGURE 1.** Crystal structure of Fn\textsuperscript{III}7B89 and comparison with other known structures of Fn fragments. A, schematic representation of the Fn\textsuperscript{III}7B89 molecule in the asymmetric unit displayed with a translucent surface. ED-B is highlighted in magenta. B, comparison of the domain boundaries and relative domain orientations between Fn\textsuperscript{III}7B89, Fn\textsuperscript{III}B8 (PDB code 2GEE), and Fn\textsuperscript{III}7–10 (PDB code 1FNF). Domain Fn\textsuperscript{III}B8 is shown in the same orientation in all three structures. To indicate the spatial rotation of each domain, both sheets of the β-sandwich have been colored differently (gray, a-b; e-blue/magenta, g-f-c-c’). C, illustration of pairwise differences in the linkage between individual adjacent domains for Fn\textsuperscript{III}7B89 and Fn\textsuperscript{III}7–10 (yellow) or Fn\textsuperscript{III}B8 (green).
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TABLE 2
Analysis of the domain boundaries in Fn

\[ \text{Rotation}^a, \text{Tilt}^b, \text{BSA}^c, N_{\text{NSB}}^d, N_{\text{NSB}}^e \]

| Domain | Rotation | Tilt | BSA | N_{\text{NSB}} | N_{\text{NSB}} |
|--------|----------|------|-----|-------------|-------------|
| Fn\text{III}7/ED-B | 55 | 37 | 387/200 | 0 | 1 |
| ED-B/Fn\text{III}8 | 133 | 71 | 201/200 | 0 | 1 |
| Fn\text{III}8/Fn\text{III}9 | 166 | 56 | 203/188 | 1 | 1 |
| Fn\text{III}9/Fn\text{III}10 (PDB code 1FNF) | 112 | 51 | 249/269 | 1 | 1 |
| Fn\text{III}8/Fn\text{III}9 (PDB code 1FNF) | 158 | 50 | 25/244 | 3 | 1 |
| Fn\text{III}8/Fn\text{III}10 (PDB code 1FNF) | 42 | 16 | 184/160 | 4 | 2 |
| ED-B/Fn\text{III}9 (PDB code 2GEE) | 89 | 58 | 215/209 | 0 | 1 |

| a | Rotation required to superimpose two adjacent Fn domains. |
| b | Tilt angles represent the acute angle of the long axes between adjacent domains (34). The long axes of the four domains in Fn\text{III}7–9 are defined by the Co positions 1201–1239, 1292–1330, 1384–1421, and 1475–1511, respectively. |
| c | Values represent the surfaces buried at each domain at the respective interface as calculated with PISA. |
| d | Number of hydrogen bonds at the domain interface. |
| e | Number of salt bridges at the domain interface. |

gated previously). In contrast, the most C-terminal Fn\text{III}8–Fn\text{III}9 domain linkage, in common between Fn\text{III}7B89 and Fn\text{III}7–10, appears conserved (Fig. 1, B and C; Table 2).

Compared with the direct Fn\text{III}7–Fn\text{III}8 connection in Fn\text{III}7–10, the consecutive Fn\text{III}7–ED-B and ED-B–Fn\text{III}8 linkages on both sides of the ED-B insertion in Fn\text{III}7B89 cause an additional right-handed twist, resulting in rotations of 59° and 28° along the string of Fn\text{III}11 domains if ED-B is present. Thus, Fn\text{III}7 and Fn\text{III}8 become twisted with respect to each other by almost 90° upon insertion of ED-B. Also, the tilt angles of the Fn\text{III}7/ED-B and ED-B/Fn\text{III}8 domain pairs in Fn\text{III}7B89 are significantly different from the one of Fn\text{III}7/Fn\text{III}8 in Fn\text{III}7–10 (Table 2). Unexpectedly, comparison of the ED-B–Fn\text{III}8 linkage between the expected contact interface and Fn\text{III}7B89 and Fn\text{III}8x-ray structures revealed significant deviations in their mutual domain rotation and tilt, too, suggesting that this particular domain interface may not be very tight. However, one should keep in mind that many residues differ in the published Fn\text{III}B8 x-ray structure from the natural ED-B sequence (31).

The buried surface area (BSA) at each of the domain interfaces in Fn\text{III}7B89 is ~200 Å². This is comparable with the interface in the Fn\text{III}8B x-ray structure. On the other hand, the Fn\text{III}7/Fn\text{III}8 and Fn\text{III}8/Fn\text{III}9 domain interfaces in the Fn\text{III}7–10 structure are larger, with values of ~250 Å² (Table 2). Furthermore, the interfaces Fn\text{III}7/ED-B and ED-B/Fn\text{III}8 in Fn\text{III}7B89 both include just one salt bridge but no hydrogen bond, indicating rather loose interdomain packing. This is supported by the significantly higher crystallographic B values of domains ED-B and Fn\text{III}8, which is in line with an increased rotational freedom of this internal Fn segment. In contrast, the apparently tighter domain interfaces Fn\text{III}7/Fn\text{III}8 and Fn\text{III}8/Fn\text{III}9 in Fn\text{III}7–10 involve one and three hydrogen bonds, respectively, in addition to one conserved salt bridge each.

Quaternary Structure of Fn\text{III}7B89 Dimer—Although the recombinant Fn\text{III}7B89 fragment was purified initially as a seemingly monomeric protein, as revealed by SEC under high salt conditions, the intimate contacts between pairs of Fn fragments within the crystal lattice indicated the presence of a biologically relevant dimer (Fig. 2). Indeed, Fn\text{III}7B89 forms an extended tight head-to-tail contact with a symmetry-related molecule, involving altogether 34 hydrogen bonds, two salt bridges, and a BSA of 1848 Å² on each monomer, resulting in a complexation significance score of 1.0 as calculated by PISA (51). Also, the shape correlation value of 0.76 calculated with the Sc algorithm (54) suggests physiologically relevant protein–protein complex formation. Notably, the 2-fold symmetry axis of the extended dimer interface runs perpendicular to the long molecular axis of each Fn\text{III}7B89 fragment and coincides with the crystallographic b axis; thus, the molecular morphology of the Fn fragment dimer also determines the macroscopic crystal shape (cf. Fig. 2D).

The Fn dimer interface can be subdivided into two main areas: (i) the one between Fn\text{III}7 and Fn\text{III}9 and (ii) the one between ED-B and Fn\text{III}8 (the prime indicating a domain from the symmetry-related neighbor; Fig. 2B). Overall, the interface is dominated by the first area, whereas the second area, involving ED-B, contributes a smaller but decisive part. The unprecedented elongated and tight contact between two large Fn segments observed in this x-ray analysis can be attributed to the mutual orientation between domains Fn\text{III}7 and ED-B as well as local conformational changes of the CC' loops in domains Fn\text{III}7 and Fn\text{III}9. In particular, the orientation of the domains Fn\text{III}7 and ED-B with respect to each other, as detailed above, and the conformational change in the CC' loop of Fn\text{III}7 create a groove that accommodates the CC' loop of domain Fn\text{III}9 from the other dimer subunit (Fig. 2A).

Fn\text{III}7B89 (excluding the His₉ tag) has a pl of 4.5 as calculated with Protparam (38), thus rendering it acidic overall. However, the negative charge is not evenly distributed across the molecule: ED-B and Fn\text{III}9 are the most and the least acidic domains, respectively, with individual pl values of 3.6 and 6.6 (Fig. 2C). Thus, the head-to-tail association of two Fn\text{III}7B89 molecules is to some extent driven by electrostatics. Patches with positive counter-charge are found on domains Fn\text{III}8 and Fn\text{III}9, whereas domains Fn\text{III}7 and ED-B appear almost entirely negatively charged.

A more detailed biochemical analysis by SEC further supported the notion that Fn\text{III}7B89 can form a dimer in solution. To this end, the Fn fragments Fn\text{III}7B89 and Fn\text{III}7–10 (lacking ED-B) were expressed and purified under similar conditions and, along with Fn\text{III}7B89, subjected to analytical SEC at different ionic strengths (Fig. 3). Notably, a decrease of the ionic strength from 300 mM to 140 mM or even 60 mM NaCl, always in the presence of 20 mM HEPES/NaOH buffer at pH 7.5, led to a significant increase in the apparent molecular size detected for Fn\text{III}7B89 and also for Fn\text{III}8 but not for Fn\text{III}7–10. A tendency to form species with elevated molecular mass was also confirmed for Fn\text{III}7B89 in AUC experiments (Fig. 3B). Consequently, insertion of ED-B into Fn fragments generally promotes dimer formation under conditions of low to physiological ionic strength, such as applied here for protein crystallization (see “Experimental Procedures”), which suggests a significant energetic contribution from electrostatic interactions, in addition to the pronounced shape complementarity.

Contrasting with the extended contact interface observed here in the crystallized dimer of Fn\text{III}7B89, the previously described x-ray structure of Fn\text{III}7–10 revealed only local, domain-wise interactions with a clearly different overall orientation of the individual Fn fragments. Indeed, the largest pack-
ing contact in the FnIII7–10 crystal structure buries a surface area of only 693 Å² and shows a PISA complexation significance score of 0.0 (Fig. 2, D and E). Taken together, it appears that the insertion of ED-B between Fn domains 7 and 8 leads to unique intermolecular interactions, which promote a new quaternary structure. The resulting Fn dimer is characterized by two novel features, the distinct tilt and rotational angles between domains along the chain and the formation of a groove between domains FnIII7 and ED-B that allows accommodation of the CC‘ loop of domain FnIII9 from the opposite subunit. This spatial arrangement has obvious implications for the overall macromolecular structure of Fn, which will be discussed below.

DISCUSSION

Despite numerous investigations in the past (14, 55), the specific physiological role of ED-B in oncofetal splice forms of fibronectin has remained unclear, especially as ED-B itself is not known to confer distinct cell-adhesive properties. Furthermore, previous structural analyses, as well as the present study, have revealed a similar fold for ED-B as for its neighboring FnIII type domains, such that its insertion into the long chain of mutually homologous Fn repeats was not expected to result in a gross effect on the macromolecular structure.

However, the crystal structure of the FnIII7B89 fragment investigated here provides two unexpected findings. First,
insertion of ED-B leads to a strong twist in the longitudinal orientation of individual FnIII repeats due to its rather short linkers and well defined interfaces with the neighboring domains. Together with the translational shift caused by the additional module itself, there is a strong effect on the global Fn structure. Second, after insertion of ED-B the central segment of Fn can form a tightly packed head-to-tail homodimer with an extended buried surface of 1848 Å² for each FnIII7–B89 monomer. This represents a unique feature of this crystal structure as compared with all previous x-ray and NMR analyses of various Fn fragments. The novel quaternary structure seems to be a consequence of the rotational/translational effect of the ED-B insertion mentioned above together with a pronounced shape complementarity in the arrangement of Fn domain pairs 7/9, B/8, 8/B, 9/7 as well as locally opposing charges and resulting electrostatic attraction.

For a more global interpretation of the effects of dimer formation on the Fn quaternary structure and its influence on potential interactions with cellular receptors, an extended molecular model for the dimeric FnIII7B8–10 fragment was generated by incorporating structural information from the two-domain moieties FnIII7–10 of the previously crystallized FnIII7–10 fragment (34) into our FnIII7B89 crystal structure. Remarkably, the relative orientation of domains 9 and 10 from the monomeric FnIII7–10 structure appeared nicely compatible with the FnIII7B89 dimer. As a result, the extended structural model revealed that the RGD loop in FnIII10 and the adhesion synergy region, comprising nine surface residues including six Arg (21, 22), shared by domains FnIII9 and FnIII10, which are both recognized by various integrins, remain accessible on the dimer surface and are oriented toward the same side (Fig. 4). On the other hand, the sterically demanding N-glycosylation site of FnIII7 at residue 1244 (56) points into the opposite direction. Within this dimeric assembly the RGD loops and synergy regions show pairwise separations by 140 Å and ~100 Å, respectively. Thus, this Fn dimer can bind two integrins at the same time, which may lead to receptor clustering on the cell surface and trigger downstream signaling (Fig. 4B).

The biochemical relevance of Fn homodimerization observed in the crystal structure was confirmed in solution via SEC and also AUC of recombinant Fn fragments, which revealed increasing dimerization tendency with decreasing ionic strength depending on the presence of ED-B. Although the energetic driving force of this dimerization for the short Fn fragments investigated here seems to be moderate, one should keep in mind that corresponding interactions are considerably enhanced, as a consequence of avidity and/or cooperativity effects, if multivalency comes into play. Indeed, this should be the case for full size Fn, which naturally forms a covalently disulfide-linked tail-to-tail dimer (Fig. 4C). Under these circumstances, insertion of ED-B may promote a homotypic interaction, which, due to the opposite direction of the Fn monomer chains involved, should result in the well known supramolecular fibril formation. In fact, this hypothesis is supported by several previous experimental data.

Fn self-association has been proposed before based on electron microscopic and biochemical findings. Distance measurements on Fn fibrils containing labeled ED-A suggested an over-

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**FIGURE 3. Biochemical analysis of FnIII7B89 dimerization.** A, apparent molecular sizes measured by analytical SEC on a Superdex 200 10/300 GL column for the recombinant fragments FnIII7B8, FnIII7B89, and FnIII7–10 at different ionic strengths of ~80, 160, and 320 mM NaCl, all in the presence of 20 mM Hepes/NaOH, pH 7.5. FnIII7B8, FnIII7B89, and FnIII7–10 have theoretical (monomeric) molecular masses of 31.5, 39.9, and 41.1 kDa, respectively. Due to their strongly asymmetric molecular shapes these Fn fragments exhibit enlarged hydrodynamic volumes and, thus, show increased apparent molecular sizes of 49, 72, and 72 kDa, respectively, under high salt conditions. Upon lowering the NaCl concentration, the apparent molecular sizes of FnIII7B8 and FnIII7B89 increase significantly by 33 and 17% (to 65 and 84 kDa), respectively, in contrast to FnIII7–10, hence indicating dimer formation. The moderate increase in size is in line with the calculated radii of gyration amounting to 45.4 Å for the FnIII7B89 dimer versus 43.4 Å for the monomer (52). B, apparent molecular weight measured by AUC for FnIII7B89. The sample (12 μM protein solution at 20 mM NaCl and 20 mM Hepes/NaOH, pH 7.5) was centrifuged at 20 °C and 23,000 rpm in a Beckman Optima XL-I analytical ultracentrifuge with Ti-60 rotor until the concentration gradient, which was monitored via UV absorption at 290 nm, reached equilibrium. Curve fit of the data using a one-species model led to a calculated mass of 59.9 ± 0.7 kDa, which is by 50% larger than the mass of the monomer (cf. A).
lapping arrangement of the amino termini of adjacent Fn (tail-to-tail) dimers with an ED-A spacing of 42 nm (57).

Investigation of the self-association propensity of Fn fragments FnIII2–14, encompassing almost the entire cell-binding segment of Fn, as well as FnIII2–14/H11001A and FnIII7–14/H11001A, both containing ED-A, via AUC demonstrated homodimerization at low ionic strengths. FnIII domains 2–3 and 12–14 within FnIII2–14 were identified as binding partners (58), which was interpreted as a local (intra-chain) association, promoting an overall compact Fn conformation.

In the light of these published experimental data our present structural findings suggest a new intriguing model for overall Fn head-to-tail association (Fig. 4C), also incorporating ED-A, that explains the macromolecular fibril formation. In this model, one chain of the extended disulfide cross-linked tail-to-tail Fn dimer makes contact to a neighboring Fn molecule via multiple interaction sites that are located alongside the chain of Fn domains, with the FnIII7B89 dimer at the center, which synergistically promote an overall head-to-tail association.

As seen from the crystal structure of the FnIII7B89 dimer, chain association is driven by electrostatic attraction between oppositely charged regions. In fact, the whole central part of the cell-binding segment of Fn shows a dipolar character, ranging from the most acidic domains FnIII2 and FnIII3, over neutral FnIII10, to the most positively charged domains FnIII12 to FnIII14 (58). Thus, insertion of ED-B should stabilize an overall head-to-tail dimerization of Fn, which, in principle, would also be possible on the basis of the previously described interactions between FnIII2–3 and FnIII12–14 (58), albeit in an intermolecular manner and extending along the entire FnIII2–14 chain. Indeed, a stabilizing effect of ED-B on Fn matrix assembly is consistent with cell culture data (59). Moreover, our model of Fn polymerization is compatible with findings of further interactions between different Fn parts described in the literature (cf. Fig.
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4C). FnIII12–14 (60) and FnIII1–2 (12, 61) have been identified as binding partners of the N-terminal portion of Fn, in particular Fn1–5. In addition, a zinc-mediated dimeric association of the five-domain Fn fragment Fn6-FnIII1–2-FnIII7–8 has been reported (62).

During the initial phase of de novo fibril assembly an elongated conformation of Fn is required, particularly to ligate integrins when binding to the cell surface (55). In line with that, dimerization of FnIII segments should impose a more rigid and extended conformation on Fn chains rather than a compact shape. In contrast, a compact conformation of the covalent tail-to-tail Fn dimer has been postulated for its plasma form, wherein both chains adopt a plaited overall structure, involving intramolecular contacts between FnIII domains 12–14 and 2–3 (58). It appears that this compact structure cannot efficiently ligate integrins (2, 58) despite its rather high plasma concentration of up to 0.3 g/liter (63).

Interestingly, ED-A and ED-B are absent in this soluble isoform of Fn (64); in fact, the occurrence of ED-A in plasma Fn is linked to thrombosis (65). These findings again suggest that insertion of one or both extra domains into Fn promotes a quaternary structure that is specific for the tissue-associated fibrillar form as well as corresponding functions of Fn in the ECM. Notably, genetic evidence points toward a mutually compensating function of the two extra domains, as single ED-A or ED-B null mice show fairly normal development whereas mice lacking both exons exhibit vascular defects leading to early death at embryonic day 9.5/10.5, despite normal Fn expression (59, 66, 67). Indeed, an ED-A mediated dimerization (possibly occurring in a different domain register) was measured for the fragment FnI6-FnII1–2-FnI7–8 has been reported (62).

As part of the ECM, Fn with its complex domain structure promotes cell adhesion, migration, growth, and survival by interacting with the integrin α5β1 (68), which is considered the pivotal integrin for vascular development (69). During corneal neovascularization (70) and tumor angiogenesis (71) expression levels of ED-B and ED-A containing Fn isoforms as well as the α5β1 integrin are significantly up-regulated, indicating that both components directly control angiogenic processes. This might also involve cross-talk between α5β1 and VEGF-R2, for which enhanced signaling has been described when its ligand VEGF-A is bound to cell-associated FnIII domains (5).

As noted above, dimerization of the cell-binding segment of Fn influences the spatial arrangement of known integrin α5β1 interaction sites in FnIII domains 9 and 10 (Fig. 4). Although insertion of ED-B leaves the individual integrin binding sites intact, the dimerization leads to exposure of a pair of these sites on the same face of the macromolecule, ready for interaction with receptors on the cell surface (22). Therefore, we propose that splicing of ED-B during angiogenesis may lead to enhanced physical clustering of integrins, which is a prerequisite for the formation of extracellular Fn fibrils (1) as these do not spontaneously polymerize in vivo. This process could also involve other integrin or non-integrin receptors that interact with Fn (8).

Taken together, the head-to-tail homo-dimer promoting effect of ED-B, and probably likewise of ED-A, on oncofetal Fn splice isoforms observed in our crystallographic study allows novel interpretation of previous data from biochemical and cell biology experiments, suggesting indirect impact of the extra domain(s) on macromolecular Fn assembly. This hypothesis should stimulate further investigations in this area.

Acknowledgments—We are grateful to H. P. Erickson (Duke University Medical Center, Durham, NC) for providing the plasmid pET11b-FN7–10. We thank Uwe Müller and Manfred Weiss for technical support at BESSY MX beamline 14.1 (Berliner Elektronenpeicherrung-Gesellschaft für Synchrotronstrahlung, Berlin, Germany, and Free University Berlin at BESSY).

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