The Compact Conformation of Fibronectin Is Determined by Intramolecular Ionic Interactions*

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Fibronectin exists in a compact or extended conformation, depending upon environmental pH and salt concentration. Using recombinant fragments expressed in bacteria and baculovirus, we determined the domains responsible for producing fibronectin’s compact conformation. Our velocity and equilibrium sedimentation data show that FN2–14 (a protein containing FN-III domains 2 through 14) forms dimers in low salt. Experiments with smaller fragments indicates that the compact conformation is produced by binding of FN12–14 of one subunit to FN2–3 of the other subunit in the dimer. The binding is weakened at higher salt concentrations, implying an electrostatic interaction. Furthermore, segment FN7–14+A, which contains the alternatively spliced A domain between FN11 and 12, forms dimers, whereas FN7–14 without A does not. Segment FN12–14+A also forms dimers, but the isolated A domain does not. These data imply an association of domain A with FN12–14, and the presence of A may favor an open conformation by competing with FN2–3 for binding to FN12–14.

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embryogenesis, wound healing, and tumor progression (7). Manabe et al. (20) showed that inclusion of A augmented the binding of fibronectin to α5β1 integrin as well as the spreading of cells on fibronectin. The activity of A was only realized in the context of intact fibronectin because the isolated A domain had no augmentation activity. These data suggested that A produced a conformational change in the compact form of fibronectin that exposed the integrin binding site.

We sought to determine the domain(s) responsible for plasma fibronectin’s compact conformation. We show here that FN12–14 is necessary to mediate fibronectin intersubunit dimerization and interacts with FN2–3. Similarly, the A domain is shown to interact with FN12–14, providing a structural mechanism for the functional effects of the A domain.

EXPERIMENTAL PROCEDURES

Materials—Thromlysin (protease, Type X), gelatin-Sepharose, and heparin-agarose were obtained from Sigma; ExpandTM High Fidelity PCR1 polymerase and dispase I from Roche Molecular Biochemicals, Pfu polymerase from Stratagene (La Jolla, CA), and Superose 6, Mono S, Mono Q, Resource Q, and cyanogen bromide-activated Sepharose 4B chromatography media from Amersham Pharmacia Biotech (Sweden).

Proteolysis of Bovine Plasma Fibronectin—Bovine fibronectin was purified from citrated serum by elution from a gelatin-Sepharose column with 4 M urea, according to standard procedures (21). For experiments with nonpurified fragments, fibronectin was incubated with 5 μg/mg thromlysin in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2 mM CaCl2, and 0.2% NaN3 (digestion buffer) for 4 or 24 h at room temperature. Four-hour digestion resulted in major fragments of 150 kDa (FN2–15+V) and 140 kDa (FN2–14) whereas 24-h digestion gave major fragments of 140 kDa (FN2–14) and 110 kDa (FN2–11) (22). Digestion was terminated by adding EDTA to 5 mM. For purification of FN2–11, plasma fibronectin was digested overnight with thromlysin. This mixture was then applied sequentially to heparin-Sepharose and gelatin-Sepharose affinity columns, with FN2–11 appearing in the flow-through from both columns. FN2–11 was dialyzed against 1 mM PIPES, pH 7, 20 mM NaCl, frozen in liquid N2, and stored at -70 °C until use. For purification of FN2–14, plasma fibronectin was incubated with 5 μg/mg Dispase I for 1 h in digestion buffer. This mixture was passed over a heparin-agarose column and eluted with 500 mM NaCl. After dialysis against 20 mM Tris, pH 8.0, peak eluted fractions were applied to a Resource Q column and eluted with a 0–500 mM linear NaCl gradient. FN2–14 was dialedyzed against 1 mM PIPES, pH 7.0, 20 mM NaCl, frozen in liquid N2, and stored at -70 °C until use. The identities of proteolytic fragments were confirmed by Western blotting.

cDNA and Expression Vector Construction for Recombinant Fibronectin Fragments—Complete cloning details are available upon request. Standard molecular biological procedures were used (23). All bacterially produced fragments were expressed by inserting PCR fragments into the pET11b expression vector (Novagen, Inc., Madison, WI). PCR reactions used ExpandTM High Fidelity polymerase. A human fibronectin cDNA (hFN-IIFull), spanning from the NsiI site to the SacII site in the fibronectin cDNA, was prepared by several ligations of fragments derived from the following cDNAs: pFH1, pFH154, pFH134, and IF10 (24, 25). All ligations and the final hFN-IIFull construct were verified by restriction analysis. hFN-IIFull was used as a PCR template for bacterially expressed fibronectin fragments not containing the A domain. All forward primers contained a Ndel site just upstream of the first codon for ligalion into pET11b. Reverse primer for FN2–14 contained an in-frame stop codon followed by a BamHI site; because there is a BamHI site in FN9, reverse primers for FN7–14, FN4–14, and FN2–14 contained an in-frame stop codon followed by a Ndel site. Forward primer for FN2–11 also contained a SacII site upstream of the Ndel site, whereas FN2–11 reverse primer contained an in-frame stop codon followed by a Smal site. Because the reverse primer for FN2–11 did not contain an Ndel site, FN2–11 PCR product was first cloned into pGEM5 as an SmalI-SmalI fragment and then recovered from the vector as an Ndel-Ndel fragment. FN2–14 was cloned into pET11b as a Ndel-BamHI PCR fragment. FN7–14, FN4–14, FN2–11, and FN2–14 were cloned as Ndel-Ndel PCR fragments; the correct orientation was selected by restriction mapping. FN7–10 was produced as described previously (3).

Expression vectors containing the A domain were made by replacing a fragment between FN7 and FN12 in the FN7–14 expression vector with a fragment containing the FNA coding sequence. A silent SpeI site was introduced into FN11 during the cloning procedures. To produce FN2–14+A, an FNA-containing fragment from FN7–14+A was cloned into the FN2–14 expression vector. FNA and FN12–14+A expression vectors were produced by PCR amplification using FN2–14+A expression vector as template.

We used a baculovirus expression system to produce full-length wild-type fibronectin (rFN wild-type), fibronectin in which FN-III domains 12–14 were deleted (ΔFN12–14), and fibronectin in which FN12–14 had been replaced by tenasin C FN-III domains A1–A3 (FNTNA1–A3). These constructs were produced from a full-length chimeric rat/human cDNA in pVL1393 baculovirus expression vector (a gift of Dr. Jean Schwarzbauer, Princeton University). In this vector, the first five type I repeats are rat (from nucleotides 186–1152 as in GenBankTM accession number X15906) whereas the remainder of the
sequence is human (from nucleotides 864–7031 as in GenBank™ accession number A14133). Production of pFN12–14 fibronectin in pVL1393 involved two PCR amplifications using Pfu polymerase to generate an SpeI site (encoding amino acids TS) replacing FN12–14, followed by several ligation steps. Restriction analysis was used to verify each cloning step as well as the final vector. FNTNA1–A3 was constructed by inserting an SpeI PCR fragment (using a human tenascin C template and Pfu polymerase) containing FN-III domains A1–A3 from human tenascin into the SpeI site of ΔFN12–14. This resulted in an insertion of the 6-base pair SpeI sequence at both ends of TNA1–A3.

**Purification of Recombinant Fibronectin Fragments**—Two types of sedimentation experiments were performed with recombinant fibronectin fragments. The first type, velocity sedimentation, does not require highly purified preparations because the data are analyzed by examining SDS-PAGE gels. However, the second type, equilibrium sedimentation, does require highly purified samples to obtain reliable data. Some of the proteins in this study (FNA, FN12–14, FN12–14-A, and FN4–14) could not be produced with sufficient purity to use for equilibrium sedimentation analysis.

All proteins expressed in BL21 cells were soluble; however, the highly expressed proteins, FN12–14, FN12–14-A, and FNA, contained significant amounts of insoluble protein after bacterial lysis. Proteins were purified by ammonium sulfate precipitation from bacterial supernatants followed by combinations of Resource or Mono Q, Mono S, and gel filtration chromatography. A complete description of purification steps is available upon request. The amounts of protein recovered varied with FN12–14 being the highest (yield of 2.5 mg/ml) and FN4–14 the lowest (yield of 0.5 mg/ml).

Fibronectins expressed in baculovirus were prepared by cotransfection of S9 cells with the fibronectin-containing pVL1393 and Baculo Gold (Pharmingen, San Diego, CA) vectors. Culture supernatants containing recombinant virus were used to infect High Five cells grown in Express Five media (Life Technologies, Inc.). High Five cells do not produce endogenous fibronectin. After culturing for 3 days, fibronectins were purified from the culture supernatant by gelatin-Sepharose chromatography (21).

**Sedimentation Analysis**—Apparent sedimentation coefficients (s) were determined by velocity sedimentation through 5-ml 15–40% glycerol gradients. Gradients were prepared using 1 mM PIPES, pH 7.0, and contained either 20 mM NaCl (low salt) or 200 mM NaCl (high salt). These salt concentrations produce near maximal changes in fibronectin sedimentation through glycerol gradients (11). Each gradient was overlaid with 200-μl samples containing a fibronectin fragment at a concentration between 200 and 500 μg/ml. Ovalbumin (3.5 S), bovine serum albumin (4.9 S), aldolase (7.3 S), and/or catalase (11.3 S) were included as internal standards. The samples were centrifuged between 34,000 and 50,000 rpm (depending upon the expected s value of the various fibronectin fragments) for 16 h in a Beckman SW-50.1 rotor at 20 °C. Following collection of 13 fractions and SDS-PAGE analysis, s values were determined by comparing the sample peaks to the standard peaks. For proteins expressed in baculovirus, sedimentation fractions were analyzed by Western blotting to detect fibronectins, in addition to silver staining the gel. s values determined in different runs were reproducible to about ± 0.2 S.

Equilibrium sedimentation was performed in a Beckman XL-A analytical ultracentrifuge. 200-μl samples, containing purified fibronectin fragments at concentrations between 200 and 400 μg/ml in 1 mM PIPES, pH 7.0, with either 20 mM NaCl (low salt) or 500 mM NaCl (high salt), were used. Because glycosylation has an effect similar to salt on fibronectin sedimentation (2, 27), the NaCl concentration used was higher than used for velocity sedimentation (200 mM). Sedimentation was performed between 5000 and 10,000 rpm at 4 or 20 °C. Base lines, obtained by centrifuging samples at high speed, were subtracted from the measurements. The resulting absorbance profiles were fit to a second-order polynomial curve as internal standards. The samples were centrifuged at 60,000 rpm for 16 h in an SW-50.1 rotor at 20 °C. Following collection of 13 fractions and SDS-PAGE, s values were determined by comparing the sample peaks to the standard peaks. For proteins expressed in baculovirus, sedimentation fractions were analyzed by Western blotting to detect fibronectins, in addition to silver staining the gel. s values determined in different runs were reproducible to about ± 0.2 S.

**RESULTS**

**Sedimentation of Bovine Plasma Fibronectin Proteolytic Fragments**—In trying to determine which portions of fibronectin mediate its compact conformation, our initial experiments examined the sedimentation of plasma fibronectin proteolytic fragments generated by thermolysin or dispase I. Thermolysin digestion of bovine fibronectin produced major bands of 110 kDa, 140 kDa, and 150 kDa, which represent FN2–11, FN2–14, and FN2–15+V, respectively, whereas dispase I digestion resulted in primarily FN2–14 (22). Table I shows the sedimentation coefficients for bovine fibronectin fragments FN2–11, FN2–14, and FN2–15+V determined in 1 mM PIPES, pH 7.0, buffered 15–40% glycerol gradients containing either 200 mM NaCl (high salt) or 20 mM NaCl (low salt). Purified FN2–11 and FN2–14 produced results indistinguishable from unpurified fragments. Sedimentation coefficients in high salt were similar for the three fragments but in low salt were substantially increased for FN2–15+V and FN2–14 but only moderately increased for FN2–11. Therefore, the presence of the FN2–14 domain within proteolytic fragments of FN2–14 and FN2–15+V was required to produce a substantial salt-dependent increase in sedimentation coefficient.

**Table I**

<table>
<thead>
<tr>
<th>Fragments</th>
<th>s (high salt)</th>
<th>s (low salt)</th>
<th>Δs</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN2–15+V</td>
<td>4.8</td>
<td>7.5</td>
<td>2.7</td>
</tr>
<tr>
<td>FN2–14</td>
<td>4.7</td>
<td>6.8</td>
<td>2.1</td>
</tr>
<tr>
<td>FN2–11</td>
<td>4.2</td>
<td>4.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**FN2–14 Is Required for Dimerization of FN2–14 and Interacts with FN2–3**—To determine the role of FN2–14 in the increased sedimentation coefficient of FN2–14, a selection of recombinant fragments within the FN2–14 region was produced in bacteria using the pET system. Sedimentation coefficients of the fragments (FN2–14, FN4–14, FN7–14, FN12–14, and FN2–11) in high and low salt glycerol gradients are shown in Table II. Recombinant FN2–14 showed a substantial change in s (Δs = 1.3), similar to its proteolytic counterpart (Δs = 2.1). This indicates that glycosylation is not required to produce the salt-induced sedimentation change; however, the smaller Δs value for recombinant FN2–14 suggests that glycosylation may enhance the salt effect. Similar to proteolytic FN2–11, recombinant FN2–11 showed only a small change in s (Δs = 0.5) compared with FN2–14. FN2–14 alone showed no salt-induced change in sedimentation properties. Likewise, recombinant fragments having incremental deletion from the amino terminus of FN2–14 exhibited only modest salt-induced changes in sedimentation coefficient. FN7–14 had a Δs of 0.7 whereas FN4–14 displayed a Δs of 0.5. These data suggest that FN2–3, like FN12–14, is required to produce the sedimentation change seen in FN2–14.

The salt-induced sedimentation change shown by recombinant FN2–14 could be explained by two mechanisms. First, FN2–14 may fold upon itself via an intramolecular interaction or second, may undergo intermolecular dimerization. To determine which mechanism was operating, the molecular weight of FN2–14 in low and high salt was determined by equilibrium sedimentation in 1 mM PIPES, pH 7.0, containing either 500 mM NaCl (high salt) or 20 mM NaCl (low salt) (Fig. 2). In high salt, FN2–14 had a molecular mass of 129 kDa, in good agreement with the expected molecular mass of a FN2–14 monomer. In low salt, FN2–14 had a molecular mass of 282 kDa, consistent with a dimer. Therefore, FN2–14 undergoes intermolecular bonding rather than folding upon itself in low salt. In contrast to FN2–14, the molecular masses of FN7–14 and FN2–11 indicated monomers at both high and low salt (Fig. 2).

Because our velocity sedimentation had suggested the
ably to the FN2–3 segment, in low salt, but the interaction is

**TABLE II**
Velocity sedimentation of bacterially expressed recombinant human fibronectin fragments without the alternatively spliced A domain

Measurements were obtained from 15–40% glycerol gradients containing 1 mM PIPES, pH 7.0, and either 20 mM NaCl (low salt) or 200 mM NaCl (high salt).

<table>
<thead>
<tr>
<th>Fragment</th>
<th>s in high salt</th>
<th>s in low salt</th>
<th>Δs</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN2–14</td>
<td>4.5</td>
<td>5.8</td>
<td>1.3</td>
</tr>
<tr>
<td>FN2–11</td>
<td>4.0</td>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>FN4–14</td>
<td>4.3</td>
<td>4.8</td>
<td>0.5</td>
</tr>
<tr>
<td>FN7–14</td>
<td>3.7</td>
<td>4.4</td>
<td>0.7</td>
</tr>
<tr>
<td>FN12–14</td>
<td>2.4</td>
<td>2.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**FIG. 2.** Equilibrium sedimentation of recombinant FN2–14, FN2–11, and FN7–14 in high and low salt. The apparent molecular masses of recombinant FN2–14 (A and B), FN7–14 (C and D), and FN2–11 (E and F) were determined by computer modeling as a function of concentration (absorbance at 230 nm; A_{230}) across the cell. Equilibrium sedimentation was performed in 1 mM PIPES, pH 7.0, containing either 500 mM NaCl (A, C, and E; high salt) or 20 mM NaCl (B, D, and F; low salt). In high salt, the apparent molecular mass of FN2–14 remained constant as a function of concentration at 129 kDa. In low salt, the apparent molecular mass of FN2–14 increased as a function of concentration, with an average of 262 kDa. Apparent molecular masses of FN7–14 and FN2–11 were approximately those of the monomer and were not significantly influenced by the salt concentration.

**FIG. 3.** FN2–11 affinity column. Starting material (SM) containing purified FN7–14 and FN7–10 in 20 mM Tris, pH 8.0, was passed over an FN2–11 affinity column equilibrated in 20 mM Tris, pH 8.0. Whereas FN7–10 came out in the flow-through (FT) and subsequent washing with 20 mM Tris, pH 8.0, FN7–14 bound to the FN2–11 column and was eluted with 20 mM Tris, pH 8.0, containing 500 mM NaCl. Later experiments showed that FN7–14 could be eluted at 50 mM NaCl. Proteins in the collected fractions were separated using SDS-PAGE and visualized by Coomassie Blue staining.

substantially weaker than the dimerization of full-length FN2–14.

**FN12–14 Is Required to Produce the Compact Conformation of Fibronectin**—Because the previous experiments were accomplished with portions of the fibronectin molecule, it remained to be determined what role FN12–14 performed in mediating the compact conformation within the context of the entire fibronectin molecule. Because Type I and II domains contain a number of disulfide bonds, we chose to express full-length and FN12–14-deleted fibronectin in baculovirus. Unlike native fibronectin in which only one subunit contains the V domain, this baculovirus expression produces fibronectin having V in both subunits.

Two constructs with FN12–14 deleted as well as full-length “wild-type” (rFN wild-type) were produced. In the first construct (ΔFN12–14), the amino acid sequence “TS” (rendered from an inserted SpeI site) replaced FN12–14. In the second construct (FNTNA1–A3), the segment FN12–14 was replaced with type III domains A1 to A3 from human tenasin-C. Following baculovirus expression, all samples were analyzed by silver stain SDS-PAGE under reducing or nonreducing conditions (Fig. 4). They all showed a single sharp band in the reduced lane; in nonreduced lanes, the most prominent band was at the position of a dimer. Each of the recombinant proteins showed a small fraction of monomer in the nonreduced lanes, but this was judged to be less than one-fourth of the total. These results indicate that fibronectins expressed in baculovirus were mostly intact dimers. FNTNA1–A3 migrated significantly slower on SDS-PAGE than either bovine plasma fibronectin or rFN wild-type. This is likely due to anomalous migration of FNTNA1–A3, because PCR and restriction analysis of the expression construct indicated that only one A1–A3 segment was inserted (data not shown).

These recombinant fibronectins were sedimented through glycerol gradients, and fractions were analyzed by silver stain SDS-PAGE and by immunoblotting for fibronectin. Each sample was sedimented with standards in the same tube, and the s value of fibronectin was estimated by linear interpolation between catalase (11.3 S) and bovine serum albumin (4.6 S). Sedimentation coefficients are given in Table III. Like plasma fibronectin, rFN wild-type showed the characteristic shift from 9.5 S in high salt to 12.6 S in low salt. The two mutant constructs with deleted or substituted FN12–14 domains sedimented at 8.4–8.6 S in high salt, suggesting that they have a more open conformation than rFN wild-type. Moreover, these constructs showed only a small (ΔFN12–14) or no (FNTNA1–A3) increase in s at low salt. We conclude that the compact conformation is substantially or completely inhibited for FN missing the FN-III domains 12–14 or having them replaced with FN-III domains from tenasin.

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The Alternatively Spliced A Domain Interacts with FN12–14—The experiments of Manabe et al. (20), suggesting an influence of the A domain on fibronectin conformation, prompted us to examine the effect of A on the sedimentation of fibronectin fragments. For these experiments, fibronectin fragments FNA, FN12–14+A, FN7–14+A, and FN2–14+A were expressed in bacteria. Velocity sedimentation data of the fragments is shown in Table IV. In high salt the fragments containing A sedimented the same as or slightly faster than the corresponding fragments without A (compare Tables II and IV). Remarkably, all fragments containing both FN12–14 and FNA showed a salt-induced change in $s$ much larger than for the same segments without A. However, FNA by itself did not show any $s$ value shift. The velocity sedimentation data suggested that the A domain of one subunit is binding to FN12–14 of the other to form a dimer. To address this issue, equilibrium sedimentation of FN7–14+A was performed. At a protein concentration of 30 $\mu$m in the presence of 150 mM NaCl, FN7–14+A formed dimers with weak associations ($K_D$ of 100 $\mu$m); at this salt and protein concentration, FN2–14 failed to associate (Table V). We conclude that FN fragments containing FN12–14+A form dimers and that this association appears to be of higher affinity than that between FN2–3 and FN12–14.

**FIG. 4.** Silver-stained SDS-PAGE of baculovirus-expressed fibronectins under reducing and nonreducing conditions. Fibronectins were expressed and purified as described under "Experimental Procedures." Proteins were run on a 5% polyacrylamide gel with or without 5% 2-mercaptoethanol as a reducing agent and visualized by silver staining. Under both reducing and nonreducing conditions, the majority of baculovirus-expressed fibronectins migrated predominantly as dimers. Lanes: A, bovine plasma fibronectin; B, ΔFN12–14; C, FNTNA1–A3; D, rFN wild-type.

**TABLE III**

Velocity sedimentation of recombinant fibronectins expressed in baculovirus

<table>
<thead>
<tr>
<th>Fragment</th>
<th>$s$ in high salt</th>
<th>$s$ in low salt</th>
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<tbody>
<tr>
<td>rFN wild type</td>
<td>9.5</td>
<td>12.6</td>
</tr>
<tr>
<td>ΔFN12–14</td>
<td>8.4</td>
<td>9.8</td>
</tr>
<tr>
<td>FNTNA1–A3</td>
<td>8.6</td>
<td>8.0</td>
</tr>
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</table>

**TABLE IV**

Velocity sedimentation of bacterially expressed recombinant human fibronectin fragments containing the alternatively spliced A domain

<table>
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<th>Fragment</th>
<th>$s$ in high salt</th>
<th>$s$ in low salt</th>
<th>$\Delta s$</th>
</tr>
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<tr>
<td>FN2–14+A</td>
<td>4.5</td>
<td>6.9</td>
<td>2.4</td>
</tr>
<tr>
<td>FN7–14+A</td>
<td>3.9</td>
<td>6.0</td>
<td>2.1</td>
</tr>
<tr>
<td>FN12–14+A</td>
<td>2.7</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>FNA</td>
<td>1.7</td>
<td>1.7</td>
<td>0.0</td>
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**TABLE V**

Equilibrium sedimentation of recombinant FN2–14 and FN7–14+A in 150 mM NaCl

<table>
<thead>
<tr>
<th>Fragment</th>
<th>$K_D$ (din)</th>
<th>$K_D$ (mon)</th>
</tr>
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<tbody>
<tr>
<td>FN2–14</td>
<td>136</td>
<td>No association observed</td>
</tr>
<tr>
<td>FN7–14+A</td>
<td>100</td>
<td>100 $\mu$m</td>
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</table>

**FIG. 5.** Models of proposed interactions producing the FN2–14 homodimer (A), the compact conformation of the plasma fibronectin dimer (B), and an altered conformation of cellular fibronectin containing the A domain (C). FN12–14 (hatched domains) interacts with FN2–3 (dark domains) and the amino-terminal Hep1 domain to form the compact conformation of plasma fibronectin. The FN12–14/FN2–3 contact is an intersubunit interaction (arrows with solid lines). The FN12–14/Hep1 contact is also shown as an intersubunit interaction (arrowsheads). A pronounced hinge point at the interface of FN9–10 (straight-lined domains) is also displayed as a bend in the compact conformation. This bend may inactivate the integrin binding site, or alternatively, the compact conformation may bury the integrin binding site in the interior. In cellular fibronectin, interaction of the A domain (shaded) with FN12–14 may disrupt the compact conformation by competing with FN2–3 and Hep1 (interaction denoted by arrows with dashed lines). Therefore, inclusion of A is hypothesized to free FN9–10 from the function-blocking effects of the compact conformation.

Our results indicate that the compact conformation of plasma fibronectin is maintained by a small number of electrostatic associations between distant segments of the molecule. A proposed structural model indicating the important associations is shown in Fig. 5. From the crystal structure of FN7–10 (3), each subunit is proposed to be relatively rigid with only a few hinge points located at specific domain interfaces. An intersubunit electrostatic bond between FN2–3 and FN12–14 brings together the two subunits of plasma fibronectin and effectively produces a compact conformation. Because our data result from a deletional analysis, however, we cannot exclude the involvement of domains near FN2–3 and FN12–14 (such as FN4 and FN11) contributing to the binding sites. The model also includes an interaction between the amino-terminal heparin binding (Hep1) domain and FN12–14, as suggested by Homandberg et al. (16). This interaction would reign in the amino termini of the plasma fibronectin subunits. Although two interactions assigned to the FN12–14 segment would appear to be hindered sterically, the interactions of FN2–3 and Hep1 with FN12–14 may occur on opposing faces of FN12–14 or use different FN-III domains within FN12–14.
FN2–14 formed a dimer detectable by sedimentation equilibrium, whereas the separate fragments FN2–11 and FN7–14 did not. The interaction of FN2–11 and FN7–14 could be demonstrated on an affinity column, but it was too weak to form stable dimers in the centrifuge. The stronger dimerization of full-length FN2–14 may be due to cooperativity. The separate fragments can only form a single electrostatic bond, but the full-length FN2–14 may be able to form two electrostatic bonds, with an FN2–3/FN12–14 pair at each end (Fig. 5). The cooperativity of forming two bonds simultaneously can substantially enhance the affinity (28).

It also seems that the interaction between FN2–14 fragments is stronger within an intact fibronectin dimer than it is for subunits free in solution. Thus, the fibronectin dimer remains in the compact conformation at 150 mM NaCl, whereas we found that FN2–14 was dimeric at 20 mM NaCl but monomeric at 150 mM NaCl. We suggest that the enhanced interaction within the dimer results from a higher effective concentration of FN2–14 binding epitopes to a restricted, favorable orientation of the epitopes. If the epitopes are about 30 nm apart in the fibronectin dimer, this would give an effective concentration of 15 μM for one epitope relative to the other. This concentration is higher than the 2 μM concentration of free subunits in our experiments but perhaps not enough to explain the full enhancement. Favorable orientation of the epitopes toward each other could enhance this interaction further.

Our experiments do not rule out additional contacts between FN12–14 and fibronectin domains outside of FN2–14. Indeed, an interaction between Hep1 and FN12–14 plasma fibronectin proteolytic fragments has previously been reported (16, 29), and we have included this contact in our model. In fact, this interaction would appear to bring fibronectin’s amino termini toward the central portion of the compact structure, further compressing the fibronectin molecule. The electrostatic nature of the interactions is consistent with the charges associated with the domains, as shown in Fig. 1. FN12–14 and FN5 are the only domains with an excess of positive charges, whereas FN2–3 and FNA have the highest negative charge. The electrostatic bonds that we observe here may involve FN2–3 or FNA, both highly negatively charged, bonding to the positively charged FN12–14. The heparin binding site in FN13 comprises a cluster of positively charged amino acids that form a cationic cradle on one face of the domain (30). This heparin binding site may be involved in the electrostatic interaction with FN2–3, but it is likely not the only contributor because heparin was found not to induce the extended conformation (31).

As discussed in the introduction, fibronectin’s compact conformation may suppress its interaction with integrins. Two structural mechanisms can be envisioned to suppress the interaction of plasma fibronectin (via FN9–10) with integrin: 1) stabilizing the protein by inducing a rotation in the fibronectin dimer, further compressing the amino termini toward the central portion of the compact structure, 2) rendering FN9–10 inaccessible to integrin by burying alteration of the spatial relationship of FN9 relative to FN10 (32, 33), and 3) rendering FN9–10 inaccessible to integrin by burying the only contributor because heparin was found not to induce the extended conformation.

Inclusion of the alternatively spliced A domain is known to augment fibronectin’s adhesion to integrin and its cell binding and spreading activity (20, 35). The increased activity was not due to the binding of A to cells. Rather, the data suggested that incorporation of A alters the conformation of fibronectin, producing a conformation favoring integrin binding. Manabe et al. (20) proposed that inclusion of A opens the compact conformation of fibronectin by inducing a rotation in the fibronectin subunit without disrupting any long range domain-domain interactions.

Our data suggest an alternative mechanism by which A could produce an open fibronectin conformation. A proposed model of fibronectin containing the alternatively spliced A domain is shown in Fig. 5C. In our model, FN12–14-A binds to FN12–14+A of the other subunit, displacing the interaction between FN12–14 and FN2–3/Hep1. This displacement produces an open fibronectin conformation freeing the integrin binding site from the constraints of the compact conformation. The data from Manabe et al. (20) and Hino et al. (35) are also consistent with such a model. In support of our model we show that FN7–14+A can form dimers whereas FN7–14 does not dimerize. Furthermore, FN12–14+A (but not FNA or FN12–14) exhibits a salt-induced change in sedimentation, consistent with dimerization. Because FNA does not interact with itself, the interaction between FN12–14 fragments probably involves A binding to one or two domains within FN12–14. The interaction between FNA and FN12–14 appears to be of higher affinity than that between FN2–3 and FN12–14 because FN7–14+A dimers are maintained in physiological salt, whereas FN2–14 does not form dimers in physiological salt.

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REFERENCES

26. Deleted in proof
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