The Self-association and Fibronectin-binding Sites of Fibulin-1 Map to Calcium-binding Epidermal Growth Factor-like Domains*  

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Fibulin-1 is a modular glycoprotein with amino-terminal anaphylatoxin-like modules followed by nine epidermal growth factor (EGF)-like modules and, depending on alternative splicing, four possible carboxyl termini. Fibulin-1 has been shown to self-associate as well as to bind calcium, fibronectin (FN), laminin, nidogen, and fibrinogen. To map ligand-binding sites within fibulin-1, polypeptides corresponding to various regions of fibulin-1 were expressed recombinantly and evaluated for their capacity to bind calcium, FN, or fibrin-1. A calcium-binding site(s) was mapped to EGF-like modules 5–9. A fibulin-1 self-association site was localized to EGF-like modules 5 and 6 (amino acid residues 356–440), as was a binding site for FN. The self-association interaction mediated by this pair of modules involved calcium since divalent cation chelators reduced the binding affinity of the interaction. By contrast, FN binding to EGF-like modules 5 and 6 was unaffected by the presence of divalent cation chelators. It can be concluded that EGF-like modules 5 and 6 bind calcium and mediate homotypic interaction between EGF-like modules 5 and 6 present in different fibulin-1 molecules and heterotypic interaction between EGF-like modules 5 and 6 and type III repeats 13 and 14 in FN. While additional binding sites for calcium or FN were not detected, another fibulin-1 self-association site was found within amino acid residues 30–173. However, unlike the self-association site in EGF-like modules 5 and 6, which was functional in the native protein, the amino-terminal site was cryptic and revealed only after the protein was denatured.
PCR fragment 1, Ala29 (within the signal peptide) was converted to Val.

mutagenesis by overlap extension (18) of two PCR fragments (fragment 7) into pcDNA3. FibA1–3E1–8 was made using site-directed

Fiblin-1 plasmid constructs were individually introduced into a

fibulin-1 subfragments to bind calcium was carried out using the method of

| TABLE I | Description of PCR-generated fibulin-1 fragments used to create fibulin-1 permutations |
|-----------------|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Fragment designation | Nucleotide residues | Amino acid residues | Upstream primer | Downstream primer | Construct name |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1 | 1–106i | 1–30 | 5 | 6 | FibE1–9C |
| 2 | 518–2200 | 175–683 | 8 | 1 | FibE1–9C |
| 3 | 1058–2200 | 356–683 | 3 | 1 | FibE5–9C |
| 4 | 1313–2200 | 441–683 | 1 | 1 | FibE7–9C |
| 5 | 1–174 | 5 | 7 | 1 | FibA1–3C |
| 6 | 1658–2200 | 555–683 | 8 | 1 | FibA1–3C |
| 7 | 1–1096 | 1–355 | 5 | 9 | FibA1–3E1–4 |
| 8 | 1–1594 | 1–524 | 5 | 11 | FibA1–3E1–8 |
| 9 | 1571–2200 | 521–524 | 10 | 1 | FibA1–3E1–8 |

The indicated primer designations refer to those described in Table II.

The numbers indicated correspond to residues within pcDNANeo (Invitrogen) plasmid.

The numbers indicated correspond to residues within human fibulin-1cDNA (1).

column of Sepharose CL-4B. The flow-through fraction was applied to one of two types of anti-fibulin-1 IgG-Sepharose affinity columns. 5D12 IgG-Sepharose was used to purify those fibulin-1 polypeptides that contained the carboxy-terminal fibulin-type module (e.g. FibE1–9C, FibE5–9C, and FibE7–9C fragments), whereas 3A11 IgG-Sepharose was used to purify those fibulin-1 polypeptides that contained the amino-terminal repeated anaphylatoxin domain. Bound protein was eluted with 4 M KSCN. After dialysis against Tris-buffered saline, the fibulin-1 polypeptide preparations were absorbed on heparin-Sepharose and gelatin-Sepharose to remove any contaminating FN, as described previously (19).

Gel Blot Overlay Assay—Recombiant fibulin-1 polypeptides were transferred to nitrocellulose membranes after SDS-PAGE. The membranes were treated with 3% nonfat dry milk in Tris-buffered saline and used to expose Kodak X-Omat AR film at 70 °C. The supernatant was twice (for 5 min each) with 50% ethanol. The membranes were dried and resuspended in 50 mM Tris, pH 7.5 (2 μg/ml final concentration). Pronase (Calbiochem) was added to a final concentration of 0.16 μg/ml and incubated for 8 h at 37 °C. An additional aliquot of Pronase was added to make the concen-
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RESULTS

Expression and Isolation of Recombinant Fibulin-1 in HT1080 Cells—A series of plasmid constructs were generated so as to permit expression in eucaryotic cells of six permutations of human fibulin-1. Fibulin-1 polypeptides were expressed that contained, either alone or in combination, each of the major structural elements found in fibulin-1, including the amino-terminal anaphylatoxin-like region (designated A), the EGF-like region (designated E), and the carboxyl-terminal fibulin-type module (designated C). The portion of the fibulin-1 protein that each construct encoded is schematically depicted in Fig. 1. Each plasmid was stably transfected into HT1080 cells, and the cDNA insert-encoded protein was isolated by anti-fibulin-1-Sepharose affinity chromatography. Shown in Fig. 2 is the SDS-PAGE analysis of the resulting preparations of recombinant fibulin-1 polypeptides. The typical yields ranged from 0.48 to 1.8 mg of protein/liter of conditioned culture medium. The results indicate that chemical amounts of fibulin-1 polypeptides corresponding to various regions of fibulin-1 could be derived from cell lines stably transfected with the fibulin-1 expression constructs.

Fibulin-1 EGF-like Modules 5–9 Bind Calcium—Previous studies have shown that fibulin-1 is a calcium-binding protein (4, 5). To localize the region(s) of fibulin-1 involved in calcium binding, purified recombinant fibulin-1 fragments were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with 45Ca2+ as described by Przysiecki et al. (22). As shown in Fig. 3, fibulin-1 polypeptides that contained EGF-like domains 5–9 (FibE1–3E1–8) and FibA1–3E1–8 bound radioactive calcium. Fibulin-1 polypeptides that lacked these EGF-like domains such as FibA1–3E1–4 and FibA1–3C showed no ability to bind calcium in this assay. These data indicate that the calcium-binding site(s) are contained within EGF-like modules 5–9.

Of the nine EGF-like modules present in fibulin-1, four (EGF-like modules 5–8) bear a consensus sequence for asparagine β-hydroxylation. Such hydroxylation has been associated with hydroxylated asparagine (23), produced a similar peak in its HPLC profile (Fig. 4, trace B). Because data from the

Fig. 1. Schematic diagram depicting the domain composition of the various fibulin-1 polypeptides expressed by transfected cell lines. In the nomenclature used for each polypeptide (indicated at the left), the letter A refers to the anaphylatoxin-like modules (the numbers 1–3 refer to the presence of three A modules); E refers to EGF-like modules, with the numbers that follow defining which of the nine EGF-like modules are included; and C refers to the carboxyl-terminal module, also known as the fibulin-type module. The dotted lines indicate that the intervening sequence has been deleted.

Fig. 2. SDS-PAGE analysis of preparations of recombinant fibulin-1 polypeptides. Shown in A and B are Coomassie Blue-stained gel profiles of the indicated fibulin-1 polypeptides electrophoresed on SDS-10% acrylamide gels in the presence (A) or absence (B) of reducing agent. Indicated on the left are the molecular masses (in kDa) of marker proteins.

Fig. 3. Fibulin-1 polypeptides that contain EGF-like modules 5–8 bind radioactive calcium. Shown in A is a Coomassie Blue-stained gel containing the indicated fibulin-1 polypeptides separated by SDS-PAGE (nonreducing conditions). Shown in B is an autoradiograph of a blot transfer from a duplicate gel as in A. The data shown are representative of four experiments.
enzymatic digestion of fibulin-1 could not be used to make quantitative estimates of the amount of \( \beta \)-hydroxyasparagine in fibulin-1, HPLC analysis of acid-hydrolyzed fibulin-1 was performed. The results of this analysis (data not shown) indicated that fibulin-1 contains 3 mol of \( \beta \)-hydroxyaspartate/mole of protein. Since the acid hydrolysis converts asparagine to aspartic acid and each of the consensus \( \beta \)-hydroxylation sites present in EGF-like modules 5–8 contains asparagine, the results can be interpreted to indicate that there are 3 mol of \( \beta \)-hydroxyasparagine in fibulin-1.

A Fibulin-1 Self-association Site Maps to EGF-like Modules 5 and 6—To localize the regions of fibulin-1 that mediate its self-association, the various recombinantly derived fibulin-1 polypeptides were coated on microtiter wells and tested for their ability to promote binding of solution-phase FITC-labeled fibulin-1. As shown in Fig. 5A, fibulin-1 polypeptides containing EGF-like modules 1–9, 1–8, and 5–9 (e.g. FibE1–9C, FibE5–9C, and FibA1–3E1–8) promoted binding of FITC-labeled fibulin-1. Fibulin-1 polypeptides that lacked EGF-like modules 5 and 6 (e.g. FibA1–3C, FibA1–3E1–4, and FibE7–9C) showed little or no ability to bind FITC-labeled fibulin-1. Similar binding results were obtained when radiiodinated or digoxigenin-labeled fibulin-1 was used as a probe (data not shown). In addition, when a recombinant fibulin-1 polypeptide that contained EGF-like modules 5–9 (FibE5–9C) was FITC-labeled and used as a probe in the binding assays, it was found to bind to those polypeptides that contained EGF-like modules 5 and 6, but not to the polypeptides that lacked the two modules (Fig. 5B). Taken together, the binding data indicate that a fibulin-1 self-association site is contained within EGF-like modules 5 and 6.

Considering the above-mentioned finding that EGF-like modules 5–8 also bind calcium, we evaluated the effect of divalent cation chelators (EDTA and EGTA) on the fibulin-1/fibulin-1 interaction. As shown in Fig. 5C, when the binding reactions were done in the presence of EDTA, there was a marked decrease in the binding of FITC-labeled fibulin-1 to fibulin-1 polypeptides bearing EGF-like modules 5–8. A similar magnitude of reduction in binding was obtained when EGTA was used instead of EDTA (data not shown). While the apparent affinity of fibulin-1 binding to polypeptides bearing EGF-like modules 5–8 was significantly reduced by either EDTA or EGTA, complete inhibition of binding was not achieved. The results suggest that calcium bound to EGF-like modules 5–8 plays an important role in the self-association interaction of fibulin-1.

A Cryptic Fibulin-1 Self-association Site Maps to the Amino-terminal Region—The ability of the recombinantly derived fibulin-1 polypeptides to promote binding of \( ^{125}\)I-labeled fibulin-1 in a gel blot overlay assay was next tested. As shown in Fig. 6, radiiodinated fibulin-1 bound only to those recombinantly derived fibulin-1 polypeptides that contained the amino-terminal anaphylatoxin-like domains (e.g. FibA1–3E1–4, FibA1–3E1–8, and FibA1–3C). Furthermore, a breakdown fragment present in the placenta-derived fibulin-1 preparation also did not bind \( ^{125}\)I-labeled fibulin-1 (see arrowheads in Fig.
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6). We subjected this fragment to amino-terminal sequence analysis and found that it began at Asp172, which occurs within the EGF-adjoining segment (data not shown). The fragment therefore lacks the anaphylatoxin-like domains and most of the EGF-adjoining segment and likely contains the nine EGF-like modules and the carboxy-terminal fibulin-type module. The results indicate that a fibulin-1-binding site exists within the amino-terminal portion of fibulin-1 (residues 30–173) containing the anaphylatoxin-like domains and the small EGF-adjoining segment.

The results from the gel blot overlay assays are contrary to those derived from the microtiter well binding assays, which showed that polypeptides containing the anaphylatoxin-like domains and the EGF-adjoining segment, but lacking EGF-like modules 5 and 6 (e.g. FibA1–3E1–4 and FibA1–3C), failed to promote fibulin-1 binding. Considering that the gel blot overlay assay involves SDS denaturation of the fibulin-1 polypeptides, whereas the microtiter assays do not, it can be concluded that the amino-terminal self-association site is not available in the native protein, but can be exposed following denaturation. In addition, these data suggest that the fibulin-1-binding site contained within EGF-like modules 5 and 6 is sensitive to denaturation and therefore can be considered to be a conformation-dependent binding site.

A Fibronectin-binding Site within Fibulin-1 Maps to EGF-like Modules 5 and 6—To localize the region(s) of fibulin-1 that mediates its binding to FN, the various recombinantly derived fibulin-1 polypeptides were coated on microtiter wells and tested for their ability to promote binding of solution-phase FN. As shown in Fig. 7A, fibulin-1 polypeptides containing EGF-like modules 1–9, 1–8, and 5–9 (e.g. FibE1–9C, FibE5–9C, and FibA1–3E1–8) promoted binding of FITC-labeled FN. Fibulin-1 polypeptides that lacked EGF-like modules 5 and 6 (e.g. FibA1–3C, FibA1–3E1–4, and FibE7–9C) showed little or no ability to bind FITC-labeled FN. Similar binding results were obtained when 30-kDa FN, a fragment of FN (type III modules 12–14) previously shown to contain the fibulin-1-binding site (6), was used as a probe (Fig. 7B). Likewise, when the 30-kDa FN fragment was coated onto microtiter wells, it promoted binding of a fibulin-1 polypeptide that contained EGF-like modules 5 and 6 (Fig. 7C). The results indicate that fibulin-1 EGF-like modules 5 and 6 contain a FN-binding site. The interaction between EGF-like modules 5 and 6 and FN was not inhibited by EDTA (data not shown) (6), unlike the interaction between EGF-like modules 5 and 6 and fibulin-1, which was partially inhibited by EDTA and EGTA (Fig. 5C).

Fibulin-1/Fibulin-1 Binding Is Mutually Exclusive of Fibulin-1/FN Binding—Since the fibulin-1 self-association site and FN-binding sites were found to map to EGF-like modules 5 and 6, we were interested in determining whether binding was mutually exclusive. As shown in Fig. 8A, solution-phase FITC-labeled fibulin-1 binding to immobilized fibulin-1 could be blocked by solution-phase FN. As an experimental control, fibulin-1 and fibulin-1 polypeptide-containing EGF-like modules 5 and 6 (FibE5–9C) competed for the solution-phase FITC-labeled fibulin-1 binding to immobilized fibulin-1, whereas a fibulin-1 polypeptide lacking EGF-like modules 5 and 6 (FibE7–9C) did not inhibit the binding. It was also found that solution-phase FITC-labeled fibulin-1 binding to immobilized 30-kDa FN was inhibited by solution-phase fibulin-1 or fibulin-1 polypeptide-containing EGF-like modules 5 and 6 (FibE5–9C) (Fig. 8B). The results indicate that the interaction of fibulin-1 with EGF-like modules 5 and 6 is mutually exclusive of the binding of FN to EGF-like modules 5 and 6. This may mean that fibulin-1, once bound to FN via EGF-like modules 5 and 6, would not support binding to another fibulin-1 via that site.

DISCUSSION

We have described the use of recombinantly derived fibulin-1 polypeptides to map three major ligand-binding sites within fibulin-1. We showed that the calcium binding activity of fibulin-1 is contained within EGF-like modules 5–9 and that EGF-like modules 5 and 6 (amino acid residues 356–440) bind fibulin-1 and FN. Furthermore, we showed that the amino-terminal region of fibulin-1 (amino acid residues 30–173) contains a cryptic fibulin-1-binding site that can be exposed by detergent denaturation.

The function of EGF-like modules 5 and 6 of fibulin-1 in mediating self-association and FN binding is fitting with the
fact that EGF-like modules within a number of other proteins have been shown to mediate protein/protein interactions. For example, the binding of Notch protein to Delta protein involves EGF-like modules 11 and 12 within Notch (24). An EGF-like module within laminin mediates its binding to nidogen (25). A single EGF-like module within the urokinase-type plasminogen activator mediates binding to the 60-kDa urokinase receptor (26). A pair of EGF-like modules in thrombomodulin mediates binding to thrombin (27). In addition, genetic diseases such as hemophilia and Marfan’s syndrome result from mutations of fibrillin-1 or between fibrillin-1 monomers, thereby resulting in defective assemblages of connective tissue microfibrils (31, 32). Similarly, mutations of the first EGF-like module of factor IX perturb the ability of the protein to mediate factor VIIa-dependent activation of factor X. Common to both genetic disorders is that mutations that inhibit calcium binding to individual EGF-like modules can result in the destabilization of helical structure adopted by calcium-binding EGF-like modules (28). The binding studies presented here indicate that the divalent cation-stabilized structure of EGF-like modules 5 and 6 is necessary for optimal fibulin-1/fibulin-1 binding, but not for fibulin-1/FN binding.

β-Hydroxylated asparagine/aspartic acid residues are present within calcium-binding EGF-like modules of a number of proteins including fibrillin-1 (33) and vitamin K-dependent coagulation proteins such as protein C (34), factor IX (35), and factor X (36). While a correlation exists between the presence of β-hydroxylated asparagine/aspartic acid residues and calcium within EGF-like modules, experiments with factor IX indicate that β-hydroxylation is not necessarily required for calcium binding (37). Consistent with the fact that four fibulin-1 EGF-like modules (EGF-like modules 5–8) have consensus asparagine β-hydroxylation sites (14), we found that fibulin-1 indeed contains β-hydroxylated asparagine. Since we detected only 3 mol of β-hydroxylated asparagine/mol of fibulin-1, it is apparent that not all of the EGF-like modules of fibulin-1 that contain the consensus hydroxylation sequences are substituted. Our data indicate that the calcium binding activity of fibulin-1 is likely contained within EGF-like modules 5–8, the same modules that have consensus asparagine β-hydroxylation sequences. It remains to be determined whether the EGF-like modules that bind calcium in fibulin-1 correspond with the three that contain β-hydroxylated asparagine.

EGF-like modules 5 and 6 of fibulin-1 appear to be quite versatile in that they can mediate self-association of fibulin-1 via homotypic EGF-like module/EGF-like module interaction as well as mediate fibulin-1 interaction with FN via a heterotypic interaction, presumably through the binding of EGF-like modules 5 and 6 to type III repeat(s) 13 and 14 of FN (6). Homotypic interaction involving EGF-like modules is likely similar to that which accounts for fibrillin-1/fibrillin-1 and Notch/Delta intermolecular interactions. Heterotypic interaction involving the binding of an EGF-like module to a non-EGF-like domain is similar to that which accounts for laminin/nidogen (25), urokinase-type plasminogen activator/60-kDa urokinase receptor (26), thrombomodulin/thrombin (27), and factor IX/factor VIIa intermolecular binding interactions. The fact that fibulin-1 uses EGF-like modules 5 and 6 to mediate both homotypic and heterotypic binding is a novel functional property, but it may turn out to be common to EGF-like modules in other proteins.

While a consequence of fibulin-1 binding to FN is that fibulin-1 becomes incorporated into FN-containing matrix fibers (3, 19), the significance of fibulin-1/fibulin-1 interaction is unknown. In experiments in which fibulin-1 was added to or recombinantly expressed in cells that are incapable of assembling a FN matrix, fibulin-1 was not found to become incorporated into matrix fibers (3, 19). However, with cells that are actively engaged in FN fiber assembly, fibulin-1 was found to incorporate into FN-containing matrix fibers. These data suggested that fibulin-1 associates with FN fibers, but, in the absence of FN, may not become assembled into fibrous fibulin-1 homopolymers. Rotary shadowing of fibulin-1 preparations shows dumbbell-shaped molecules (38) that fit a model of globular amino- and carboxyl-terminal domains separated by an extended stretch of repeated EGF-like modules. Whether these dumbbell-shaped molecules are monomers or dimers remains to be established. It is possible that the centrally located EGF-like modules 5 and 6 could mediate parallel or antiparallel alignments of fibulin-1 monomers that could produce dumbbell-shaped dimers. In sedimentation equilibrium experiments performed under physiological ionic strength conditions (phosphate-buffered saline), we determined a molecular mass of 150 kDa for fibulin-1, whereas under dissociative conditions (6 M guanidine HCl), a value of 60 kDa was derived.2 Using laser desorption mass spectroscopy, the conditions of which are generally considered unfavorable for preservation of noncovalent intermolecular interactions, a value of 78,842 Da was determined for the molecular mass of fibulin-1 monomer (8). Taken together, it seems apparent that under physiological conditions, fibulin-1 can form noncovalently associated dimers. Whether fibulin-1 dimers can align with one another through lateral association of EGF-like modules 5 and 6 to form arrays of fibulin-1 and whether such arrays would have physiological relevance are not known.

In addition to EGF-like modules 5 and 6, another fibulin-1 self-association site was localized within the amino-terminal region. Unlike the site in EGF-like modules 5 and 6, the activity of the amino-terminal site was masked in the native protein and required detergent denaturation for it to be revealed. This site can therefore be considered a cryptic self-association site. A cryptic self-association site has been identified within the first type III module (III-1) of FN (39–41). It has been proposed that unfolding of III-1 is a prerequisite to the polymerization of FN.

2 H. Tran and W. S. Argraves, unpublished data.
Similarly, the amino-terminal cryptic site of fibulin-1 may be important for assembly of fibulin-1 into polymers. Rotary shadowing of fibulin-1 preparations has revealed the presence of spider-shaped multimers of fibulin-1 (38). These eight to nine-legged spider-shaped structures appear to contain the previously described fibulin-1 dumbbells, centrally interconnected through terminal globular domains. It is tempting to speculate that self-association of fibulin-1 dumbbells, centrally interconnected through terminal globular domains. It is tempting to speculate that the self-association site contained within the amino-terminal region of fibulin-1 mediates the interaction involved in the formation of the spider-shaped multimers. In the case of FN, exposure of the III-1 cryptic site to initiate polymerization requires a cell-dependent action, perhaps involving integrin \( \alpha_\beta_1 \) (39). The physiological mechanism that would lead to unfolding of the amino-terminal cryptic self-association site of fibulin-1 remains to be established.

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