Fibrillin-1 and Fibulin-2 Interact and Are Colocalized in Some Tissues

(Received for publication, March 25, 1996, and in revised form, May 15, 1996)

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Microfibrils 10–12 nm in diameter are found in elastic and non-elastic tissues with fibrillin as a major component. Little is known about the supramolecular structure of these microfibrils and the protein interactions it is based on. To identify protein binding ligands of fibrillin-1, we tested binding of recombinant fibrillin-1 peptides to different extracellular matrix proteins in solid phase assays. Among the proteins tested, only fibrillin-2 showed significant binding to rF11, the N-terminal half of fibrillin-1, in a calcium-dependent manner. Surface plasmon resonance demonstrated high affinity binding with a Kₐ = 56 nM. With overlapping recombinant fibrillin-1 peptides, the binding site for fibrillin-2 was narrowed down to the N terminus of fibrillin-1 (amino acid positions 45–450). Immunofluorescence in tissues demonstrated colocalization of fibrillin-2 and fibrillin-1 in skin, perichondrium, elastic intima of blood vessels, and kidney glomerulus. Fibulin-2 was not present in ocular ciliary zonules, tendon, and the connective tissue surrounding kidney tubules and lung alveoli, which all contain fibrillin. Immunogold labeling of fibrillin-2 on microfibrils in skin was found preferentially at the interface between microfibrils and the amorphous elastin core, suggesting that in vivo the interaction between fibrillin-1 and fibrillin-2 is regulated by cellular expression and deposition as well as by protein-protein interactions.

Microfibrils have been identified as small diameter (8–12 nm) extracellular matrix fibrils found in close proximity to basement membranes (Low, 1962) and elastic fibers (Cleary and Gibson, 1983). Microfibrils can be distinguished from banded collagen fibers since microfibrils display no clear banding pattern, have relatively uniform diameters, are found in loose bundles or in bundles aggregated by elastin, and often appear hollow in cross sections. Elastic fiber microfibrils and basement membrane-associated microfibrils share at least one major component, fibrillin (Sakai et al., 1986).

Cloning and sequencing investigations have identified two different genes for fibrillin (Maslen et al., 1991; Lee et al., 1991; Corson et al., 1993; Pereira et al., 1993; Zhang et al., 1994a), which code for highly homologous proteins. Both proteins have 47 EGF-like domains, 43 of which are of the calcium-binding type, interspersed by two types of cysteine-rich repetitive elements, the “8-cysteine” domain and the “hybrid motif” (Maslen et al., 1991; Corson et al., 1993), which can also be found in the family of transforming growth factor β-binding proteins (Kanzaki et al., 1990; Morén et al., 1994; Yin et al., 1995b).

Although both fibrillins have been immunolocalized to microfibrils (Sakai et al., 1986; Zhang et al., 1994a), the specific functions of these proteins are unknown. Indications for similar but distinct functions for fibrillin-1 and fibrillin-2 in extracellular matrices have emerged from mutation analysis of their genes. Mutations in FBN1 cause the Marfan syndrome (Dietz et al., 1994), whereas FBN2 is linked to congenital contractual arachnodactyly (Lee et al., 1991; Tsipouras et al., 1992). Recently, the first fibrillin-2 mutations have been described in this disease (Putnam et al., 1995).

Electron micrographs of basement membrane regions in skin (Tsuji, 1980), lung (Low, 1961), kidney (Farquhar et al., 1961), and lens capsule (Raviola, 1971) reveal small connective tissue spaces with bundles of microfibrils that appear to intersect the lamina densa. These microfibrils intersecting basement membranes lack a visible amorphous elastic component. In some tissues like skin, continuous tracts of microfibrillar bundles may extend between basement membranes and adjacent elastic fibers. These electron microscopic observations suggest that microfibrillar proteins may interact with diverse types of extracellular matrix (ECM) molecules: components of basement membranes as well as elastic fiber components. In addition, microfibrils may interact with other components that are present in the ECM. Immunolocalization experiments have demonstrated that fibrillin is periodically arranged in microfibrils (Sakai et al., 1986) and that fibrillin periodicity in microfibrils liberated from their tissue environment is variable and extendable (Keene et al., 1991). In relaxed tissues, the fibrillin periodicity appears to match the periodic banding pattern of collagen fibers (Sakai et al., 1986). These observations suggest that fibrillin microfibrils may interact with molecules, which in turn interact with collagen fibers to stabilize the ECM.

The structure of the fibrillins contains 43 calcium-binding (cb) EGF-like domains. This type of module has been shown to mediate certain specific protein-protein interactions, for exam-
ple the clotting activity of human coagulation factor IX (Rees et al., 1988) or the interaction of protein S with complement C4b-binding protein (Dahlbäck et al., 1990). Similar tandem arrays of cb EGF-like repeats exist in the extracellular matrix proteins fibrillin-1 and fibrillin-2 and are likely to be involved in calcium-dependent binding to other extracellular ligands such as fibronectin and nidogen (Sasaki et al., 1995a, 1995b). For Drosophila Notch, which is composed of 36 EGF-like repeats (25 of which contain consensus sequences for calcium binding) (Wharton et al., 1985), calcium-dependent binding to Delta and Serrate is mediated by two tandem cb EGF-like repeats (EGF repeats 11 and 12) (Rebay et al., 1991). Therefore, it has been proposed that Notch may serve as a multifunctional molecule composed of a tandem array of discrete ligand-binding units (Rebay et al., 1991). Similarly, fibrillins may be multifunctional molecules composed of tandem arrays of discrete ligand-binding domains.

To test this hypothesis as well as to specifically test whether known candidate ECM molecules may interact with fibrillin to stabilize the integrity of certain connective tissue zones, we have performed ligand-binding assays using recombinant peptides of fibrillin-1 and determined that fibrillin-2, a novel ECM molecule (Pan et al., 1993), binds to fibrillin-1. Moreover, we show that fibrillin-2 is localized to some microfibrils, demonstrating that specific binding may also occur in vivo. However, not all fibrillin-containing microfibrils are labeled with antibodies to fibrillin-2, suggesting that functional differences exist between microfibrils in different connective tissues.

MATERIALS AND METHODS

Expression of Recombinant Proteins

Recombinant mouse fibrillin-1 variants C and D and mouse and human fibrillin-2 were prepared as described (Pan et al., 1993; Sasaki et al., 1995a, 1995b). Recombinant fibrillin-1 peptides rF6, rF11, and rF20 were produced and purified as described (Reinhardt et al., 1996). An additional recombinant fibrillin-1 construct (rF23), coding for S5-I-1399, was prepared by PCR amplifying template HFBN29 (Corson et al., 1993) with oligonucleotides N1540S (5’-GATGGTGATGAATACACTCCCCACGGAGG-3’) and N1329S (5’-CAACAAGCTGTGCTCTGTT-3’), which introduced the sequence coding for six histidine residues, a stop codon, and a translation site at the 3’ end. The 74-base pair Age-NotI fragment of the PCR product was then fused with the Age-NotI restricted expression plasmid pCis-rF11H (Reinhardt et al., 1996). A 1441-base pair Nhel-NotI fragment was then fused with the Nhel-NotI restricted pCPEAP4/2I14 (Mayer et al., 1995). The resulting plasmid was designated pCEPS-P-rF23H. The correct insertion of the insert and the sequence of the PCR-amplified DNA was confirmed by automated DNA sequencing following the manufacturer’s instructions (Applied Biosystems). Transfection of 293/EBNA cells (Invitrogen), selection of clones, and purification of the recombinant peptide was done as described for construct rF20 (Reinhardt et al., 1996).

Proteins from Tissue or Cell Culture Sources and Antibodies

Mouse laminin-1 nidogen complex (Paulsson et al., 1987), human recombinant BM-40 (Nischt et al., 1991), and pepsin-solubilized bovine or human laminins I, II, III, IV, and V (Miller and Rhodes, 1982) were prepared following established procedures. Bovine collagen XI was a gift of Dr. David Wiley, and human plasma fibronectin was of commercial origin (Behringwerke).

For extraction of extracellular matrix proteins from cell culture sources, confluent layers of normal human skin fibroblasts were first washed two times with 50 mM Tris and 150 mM NaCl, pH 7.4 (TBS) including 2 mM phenylmethylsulfonfyl fluoride and 5 mM N-ethylmaleimide (protease inhibitors). The cells and the residual extracellular layer were then sequentially extracted with 0.1 M/c2 MBS, including protease inhibitors, and 0.5% (w/v) Triton X-100 (cells) or 10 mM EDTA (extracellular layer) for 10 min at 25°C.

Polyclonal antisera against mouse fibrillin-1 and mouse and human fibrillin-2 were described and characterized previously (Pan et al., 1993; Sasaki et al., 1995a, 1995b). Monoclonal antibodies mAb 26, mAb 201, and mAb 69 were those as described (Sakai et al., 1986; Maddox et al., 1989; Reinhardt et al., 1996).

Binding Assays

The binding of different proteins to recombinant peptides was investigated by an enzyme immunoassay or a blot overlay assay. For the enzyme immunoassay, multwell plates (Costar, 96 wells) were coated with purified recombinant protein (100 ng/ml) in 100 mM Na2CO3 and 35 mM NaHCO3, pH 9.2, for 16 h at 4°C. For the blot overlay assay, recombinant fibrillin-2 (1 μg) and EDTA extract of the extracellular layer of skin fibroblasts (2 ml, precipitated with trichloroacetic acid) were separated by SDS-gel electrophoresis (5% w/v acrylamide) and then transferred onto nitrocellulose membrane (Bio-Rad) in 10 mM sodium borate, pH 9.2 (0.4 A for 45 min). Each of the following incubations was performed at 25°C and was followed by washing the wells or the nitrocellulose membrane three times with TBS including 0.025% Tween 20. Nonspecific binding sites were blocked with 5% nonfat dry milk in TBS for 1 h. The wells were then incubated with serial dilutions of the soluble ligand, and membranes, with a fixed concentration of 100 ng/ml recombinant protein for 3 h, in TBS/2% dry milk including 2 mM CaCl2. Incubation (1.5 h) with monoclonal or polyclonal antibodies against the soluble ligand (1:100–1:2000) in TBS/2% dry milk was then followed by incubation (1.5 h) with a peroxidase-conjugate of goat anti-mouse or goat anti-rabbit immunoglobulin (Bio-Rad, 1:200–1:800) in the same buffer. The color reaction of the enzyme immunoassay was developed with 1 mg/ml 5-aminosalicylic acid (Sigma) in 20 mM phosphate buffer, pH 6.8, containing 0.1% H2O2 (100 μl/well) for 3–4 min and stopped by adding 2 ml NaOH (100 μl/well). Color yields were determined at 492 nm. Nitrocellulose membranes were developed in a solution of 100 ml TBS/0.02% (w/v) H2O2 plus 20 ml of 3 mg/ml 4-chloro-1-naphtol in methanol (Bio-Rad) until bands were clearly visible.

For kinetic binding studies, the BIAcore biosensor (Pharmacia Biotech Inc.) was used. Biotinylated fibrillin-2 (50 μg/ml) was coupled in 0.1 M sodium acetate, pH 4.0, to sensor chip SAS (streptavidin chip), and then resulted in 5500 resonance units equivalent to about 5 ng/mm2 immobilized on the chip. Binding studies with soluble recombinant fibrillin-1 peptides rF11 in the range of 0.27–1.5 μM and rF6 (0.5 μM) were performed in TBS and 0.05% P20 (Pharmacia) containing either 2 mM CaCl2 or 3.4 mM EDTA. Kinetic rate constants were calculated according to Fägerstam et al. (1992) with BIAevaluation software version 2.1 (Pharmacia).

Immunofluorescence

Fetal bovine tissues were obtained from a local slaughterhouse. Human tissues were from a 16-week-old fetus, obtained according to human use protocols. The tissues were frozen in hexanes (EM sciences) and embedded in OCT (Miles). Cryosections (7 μm) were prepared using a Krystalat 1720 (Leica). The sections were air-dried, fixed in acetone (10 min, −20°C), and then rehydrated in PBS. The sections were then incubated with a mixture of a 1:100 dilution of antisera to mouse fibrillin-2 and 100 ng/ml mAb 201 or mAb 69 to fibrillin. After washing with PBS, the tissue sections were incubated with 1:50 dilutions of either phycoerythrin-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG (Sigma). The sections were mounted in Gel mount (Biomed) and photographed using an Axiopt (Zeiss) or Leitz DMRB (Leica) microscope equipped for epifluorescence.

Electron Microscopy

Section Surface Immunolabeling—Skin from the back of a 12-year-old male was obtained during surgery and processed for electron microscopy as described previously (Sakai and Keene, 1994). Fresh tissue was fixed in ice-cold 0.1% glutaraldehyde, rinsed in buffer and Tris-HCl, dehydrated in ice-cold ethanol, and embedded in Lowicryl K4M. Polymerization in a CO2 atmosphere was accomplished using UV (light at −20°C). Ninety-mm-thick sections, which included the epithelium, papillary, and reticular dermis, were mounted onto Formvar-coated slot grids and exposed to a polyclonal antisera against human fibrillin-2 diluted 1:5 in PBS, followed by a goat anti-rabbit 10-nm colloidal gold conjugate (Amersham Corp.; diluted 1:3 in Tris/HCl with 1% bovine serum albumin, pH 8.0). Grids were exposed to the electron beam prior to staining in uranyl acetate and lead citrate. Controls included samples where the primary antibody was omitted and where a primary
Protein Ligands of Fibrillin-1

Fig. 1. Binding of soluble fibrillin peptides in a solid phase binding assay to immobilized fibrulins. The curves show binding of the N-terminal fibrillin peptide rF11 to fibulin-1C (A), fibulin-1D (Y), human fibulin-2 (dashed line, ■), mouse fibulin-2 in regular binding buffer (solid line, ●) and in a 1 M NaCl containing binding buffer ( ). No binding was observed for the C-terminal fibrillin peptide rF6 to mouse (C) and human (data not shown) fibulin-2. Similar binding profiles were observed with immobilized fibrillin peptides and fibulin-2 in the soluble phase. Data points represent averages of duplicates. For clearer graphs, only the positive parts of the error bars are indicated. The experiment was repeated five times.

Miscellaneous Methods

Protein concentrations were determined in triplicate after hydrolysis (6 M HCl, 24 h, 110 °C) on a Beckman 6300 amino acid analyzer. For N-terminal sequencing, rF23 was analyzed on an automated protein sequencer (Hewlett Packard G1000S).

RESULTS

Protein Ligand Binding of Recombinant Fibrillin-1 Peptides—To identify potential binding ligands of fibrillin-1, a variety of extracellular matrix proteins including fibronectin, laminin-1, BM40 (SPARC), fibulin-1C and fibulin-1D, fibulin-2, and collagen types I, II, III, IV, V, and XI were tested in solid phase assays for binding to recombinant fibrillin-1 peptides rF11 (amino acid positions 45-1527) and rF6 (amino acid positions 1487-2871), which together span the whole fibrillin-1 molecule (Reinhardt et al., 1996). Among the proteins tested, only fibulin-2 showed significant binding to the N-terminal peptide rF11 but not to the C-terminal peptide rF6 (Fig. 1). All other ligands, including the isoforms fibulin-1C and fibulin-1D, had no or only minor affinities to the fibrillin-1 peptides. Nearly identical binding profiles of human and mouse fibulin-2 to rF11 indicate a conserved binding epitope between species, suggesting an important function of the fibrillin-1/fibulin-2 interaction (Fig. 1).

The interaction of fibulin-2 with rF11 was also analyzed by a surface plasmon resonance method to determine kinetic rates in real time (Fägerstam et al., 1992). Since the binding profiles for mouse and human fibulin-2 were nearly identical (Fig. 1) and the availability of human fibulin-2 was limited, we used mouse fibulin-2 for these experiments. Soluble rF11 in the concentration range of 0.27–1.5 μM bound in a dose-dependent manner (14–75 response units) to fibulin-2 fixed on a sensor chip (Fig. 2). The C-terminal fibrillin-1 peptide rF6 was inactive. Kinetic rate constants for the association (k<sub>on</sub> = 57.8 × 10<sup>-2</sup> ± 3.7 × 10<sup>-3</sup> M<sup>-1</sup> s<sup>-1</sup>) and dissociation (k<sub>off</sub> = 3.18 ± 0.35 × 10<sup>-3</sup> s<sup>-1</sup>) were calculated for each concentration and resulted in a dissociation constant of K<sub>d</sub> = 56.3 ± 3.5 nM for the fibrillin-1/fibulin-2 interaction. Binding was abolished when CaCl<sub>2</sub> in the binding buffer was replaced by EDTA (Fig. 2). However, when fibulin-2 was preincubated with rF11 in the CaCl<sub>2</sub>-containing binding buffer, the complex could not be dissociated by EDTA, indicating that after binding of the proteins, the calcium ions are held firmly within the binding site.

The interaction was also tested with authentic fibulin-2 and fibrillin from cell culture sources. We performed an overlay assay where one ligand is bound to nitrocellulose after electrophoresis under nonreducing conditions, and the other ligand is soluble. rF11 in the soluble phase bound to a 600-kDa band of an EDTA extract of human skin fibroblasts and to recombinant fibulin-2, which comigrated with this band (Fig. 3, left panel). No binding was observed when rF11 was omitted (Fig. 3, middle panel). The 600-kDa band was identified as authentic fibulin-2 by reaction with a 1:10,000 diluted polyclonal antibody specific for fibulin-2 (Fig. 3, right panel). A second band (290 kDa), which reacts with rF11, represents possibly another fibrillin-binding protein, which is not yet identified.
When rF11 and authentic fibrillin from serum-free medium of skin fibroblasts were immobilized onto nitrocellulose, no binding could be observed with soluble recombinant fibulin-2 (data not shown). These data suggest that the binding site in fibrillin-1 is inactivated after SDS treatment for gel electrophoresis, whereas the binding site in fibrillin-2 is not affected by SDS.

For solid phase assays with authentic fibrillin, we purified small quantities (10 μg) from 1 liter of serum-free cell culture medium by affinity chromatography on mAb 26. The eluate of the affinity column was concentrated to 1 ml and then used in the binding assay. A typical binding profile was observed with immobilized recombinant fibulin-2, whereas the control without fibulin-2 did not show binding (data not shown). However, the absolute numbers of these experiments were relatively low (maximum absorbance at 492 nm = 0.15), probably due to the low amounts of fibrillin present in the soluble phase or alternatively to partial inactivation of the fibulin-2 binding site by proteolysis. Similar results were obtained with inversed orientation of the ligands in the assay.

Localization of the Fibulin-2 Binding Site on Fibrillin-1—To narrow down the binding site for fibrillin-2 on fibrillin-1, we produced recombinantly a subdomain of fibrillin-1 (rF23) (Table I), which together with the previously described subdomain rF20 spans the whole N-terminal fibulin-2-binding peptide (rF11). The episomal expression plasmid designed was pCEPSP-rF23 coding for S^{19-489} of fibrillin-1 plus additional N-terminal (APLA; Mayer et al., 1995) and C-terminal (HHHHHHH) amino acids. The peptide was expressed in stably transfected 293/EBNA cells in amounts of 10–15 μg/ml/day. After a one-step purification on a cobalt-loaded chelating column (Reinhardt et al., 1996), the peptide showed >95% homogeneity on SDS gel electrophoresis (data not shown) with an apparent molecular mass of 63 kDa, which is slightly higher than expected from the cDNA (Table I). The observed difference is likely due to the occupation of one N-glycosylation site predicted in this peptide (position 448–450). Edman degradation of rF23 revealed a sequence 30 amino acid residues shorter than expected (R^{45}GGGGHDALKGP) (Table I). This N-terminal sequence, also observed previously for rF11 (Reinhardt et al., 1996), occurs after a consensus processing sequence for many propeptides (RXK/RR; Hosaka et al., 1991), suggesting that fibrillin-1 undergoes intracellular N-terminal processing.
glomeruli (Fig. 5C, tissue around kidney tubules (Fig. 5). Fibulin-2 and fibrillin appeared to colocalize in perichondrium (Fig. 5, served in some tissues (Fig. 5). Whether the binding site involves the binding of rF20 clearly demonstrate that the binding site for fibrillin-1/fibulin-2 interaction was determined. This is the first demonstration of a ligand-binding interaction specific for fibrillin-1.

Fibulin-2 was discovered by cDNA cloning and shown to be homologous to fibulin-1 (Pan et al., 1993; Zhang et al., 1994b). Binding studies using fibulin-1 suggest multiple interactions with other ECM molecules (Balbona et al., 1992; Brown et al., 1994; Sasaki et al., 1995a). The repertoire of ligands of fibulin-2 is similar, although not identical to that of fibulin-1; fibrillin-2 binds to fibronectin, nidogen, collagens IV and VI, and perlecan (Sasaki et al., 1995b). In our studies, fibulin-2 (mouse and human) binds to fibrillin-1 but mouse fibulin-1 does not. It remains possible that human fibulin-1 interacts with fibrillin-1.

Tissue Distribution of Fibulin-2 and Fibrillin-1—When tissues were double labeled with polyclonal antiserum to fibulin-2 and monoclonal antibodies to fibrillin, colocalization was observed in some tissues (Fig. 5). Fibulin-2 and fibrillin appeared to colocalize in perichondrium (Fig. 5A and B, arrows), kidney glomeruli (Fig. 5C and D, arrows), and blood vessels (Fig. 5E and F). In the blood vessel, fibulin-2 staining appeared to be more intense in the intima (Fig. 5E, arrowheads), whereas fibrillin staining was throughout the vessel wall and intense in the adventitia (Fig. 5F, arrows). Fibulin-2 staining was not observed in tendon (Fig. 5A, arrowheads), in the connective tissue around kidney tubules (Fig. 5C, arrowheads), in lung alveolar connective tissue (Fig. 5E), or in the ciliary zonules (Fig. 5G).

Electron Microscopic Immunolocalization—Ultrastructural localization of fibulin-2 is shown in Fig. 6. In "en bloc" immunolocalization experiments of fibulin-2, the antibody-directed gold labeling was to microfibrils at the outer periphery of the amorphous elastin core. No labeling of microfibrils positioned away from the periphery of the elastin core was noted (Fig. 6E). Immunolocalization of fibulin-2 to the surfaces of cross-sections of elastin fibrils of lightly fixed skin demonstrated that the majority of gold particulates delineate the periphery of the elastin fibrils and are located at the intersection of microfibrils with elastin (Fig. 6A–D). The relatively low amount of labeling within the elastin core is approximately equal to that seen randomly distributed on the section surface and, therefore, represents background labeling. By either the diffusion or section surface methods, elastin fibers in any location of the deep reticular dermis or close to the dermal-epidermal junction were labeled as described above.

DISCUSSION

Candidate ECM ligands (fibronectin, laminin-1, BM40/SPARC, fibrilins-1 and -2, and collagens I, II, III, IV, V, and XI) were tested for binding to recombinant fibrillin-1 peptides. One ligand, fibulin-2, was identified, and a dissociation constant (Kd = 56 nM) for the fibrillin-1/fibulin-2 interaction was determined. This is the first demonstration of a ligand-binding interaction specific for fibrillin-1.

Fibulin-2 was discovered by cDNA cloning and shown to be homologous to fibulin-1 (Pan et al., 1993; Zhang et al., 1994b). Binding studies using fibulin-1 suggest multiple interactions with other ECM molecules (Balbona et al., 1992; Brown et al., 1994; Sasaki et al., 1995a). The repertoire of ligands of fibulin-2 is similar, although not identical to that of fibulin-1; fibrillin-2 binds to fibronectin, nidogen, collagens IV and VI, and perlecan (Sasaki et al., 1995b). In our studies, fibulin-2 (mouse and human) binds to fibrillin-1 but mouse fibulin-1 does not. It remains possible that human fibulin-1 interacts with fibrillin-1.

Extensive structural studies of fibrillin-1 recombinant peptides (Reinhardt et al., 1996) have shown that the peptides utilized in these studies are fully functional and equivalent to native peptides. To demonstrate the biological relevance of these in vitro studies with recombinant peptides, we performed immunolocalization studies. Ultrastructural investigations of skin revealed fibulin-2 primarily at the interface of amorphous elastin cores and microfibrils, even though fibrillin-1 is a component of all microfibrils. These data suggest that fibulin-2 may also bind to a component of the amorphous elastin core. A similar distribution was recently demonstrated for emlin (Bressan et al., 1993). In contrast, fibrillin-1 is located within the amorphous core of elastic fibers, but not in the fibrillin-containing, elastin-associated microfibrils (Roark et al., 1995). Although more peripheral microfibrils were not labeled by fibulin-2 antibodies, these antibodies did label microfibrils at the dermal-epidermal junction. However, antibody labeling of these microfibrils was not very extensive.

Several lines of evidence suggest that fibulin-2 is a microfibril-associated protein rather than an integral structural component of microfibrils. Fibulin-2 labeling of microfibrils is not linearly periodic. Some, but not all, tissues containing microfibrils are labeled by fibulin-2 antibodies. Fibulin-2 can be extracted from tissues or cell layers with EDTA, indicating that it is not a covalently cross-linked component of microfibrils, and in vitro binding studies with fibrillin-1 peptides showed strongly reduced binding affinities in the presence of 1 M NaCl. Beaded microfibril structures, on the other hand, have been
Electron microscopic immunolocalization of fibulin-2 to microfibrils on the surface of sections of 12-year-old skin (A-D) or to neonate foreskin by the “enbloc” method (E). The majority of gold particles are associated with microfibrils located at the periphery of elastin fibrils, as seen in cross-section (A and B), tangential section (C), or longitudinal section (D and E). Little label is seen within the core of the elastin fibrils, except in areas likely to be invaginations of the elastin fiber surface (D). Only microfibrils in close vicinity of the elastin fiber are labeled. Bars: A–D, 200 nm; E, 200 nm.
identified after repeated extractions of tissues including 1 mM NaCl washes, collagenase digestions, and final extraction with guanidine-HCl (Keene et al., 1991), conditions which would be expected to dissociate fibrillin-2 from the microfibrils. These data, together with the noted absence of fibrillin-2 in tissues that are subject to strong tensile forces (e.g. tendon and ciliary zonule), suggest that the fibrillin-1/fibulin-2 interaction is not required for the structural/mechanical integrity of microfibrils.

Immunohistochemical studies and in vitro binding data demonstrate that fibrillin-1 interacts with fibrillin-2 in certain tissues (skin, perichondrium, kidney glomerulus, and the elastic intima of blood vessels) and not in others (tendon, cartilage, ciliary zonule, other areas of connective tissue in the kidney, and lung). Since fibrillin-2 is able to interact with multiple ECM molecules in in vitro binding experiments, a cellular expression, cellular deposition of molecules into the ECM, and the multifunctional nature of fibrillin-2 must regulate the in vivo protein-protein interactions of these molecules. In basement membrane regions, like the kidney glomerulus and the dermal-epidermal junction, which do not contain visible amorphous elastic cores, fibrillin-2 may bind to fibrillin-1 and basement membrane proteins (e.g. nidogen or type IV collagen) to stabilize the interaction between microfibrils and the lamina densa. The interaction of microfibrils with the lamina densa is likely not stabilized by direct binding between fibrillin-1 and the major basement membrane components (type IV collagen, laminin-1, and nidogen), since no binding between these molecules was detected in our studies.

In addition to a potential role in stabilizing interactions between structural elements of the ECM (microfibrils and elastic cores; microfibrils and basement membranes), the fibrillin-1/fibulin-2 interaction may play an important role in developmental processes. Both molecules are highly expressed in the developing heart. At early developmental stages, the most striking expression of fibrillin-1 and fibrillin-2 is in endocardial cushion tissue. In the developing mouse embryo (days 8.5–9.0), the endocardial tissue was the only place where Fn1 could be detected by in situ hybridization (Yin et al., 1995a), and at day 11, fibrillin-2 expression was found exclusively in the endocardial cushion (Zhang et al., 1995a). In the developing chick, fibrillin was detected by immunofluorescence in the endocardial layer at stage 11 and in the developing cushion mesenchyme from stage 20 on (Hurle et al., 1994). The development of the heart from a muscular tube into a chambered structure involves the transformation of endocardial cells into mesenchymal cells that form the endocardial cushion tissue; the endocardial cushion then contributes to the formation of the valves and septa of the chambered heart (Little and Rongish, 1995). The prominent coexpression of fibrillin-1 and fibrillin-2 in a transitional developing structure, the endocardial cushion, suggests that the interaction of these molecules may promote the transition of endocardial cells to mesenchyme, contribute to a specialized cushion matrix, and also permit or promote the formation of valves and septa.

The calcium-dependent nature of the fibrillin-1/fibulin-2 interaction suggests that cb EGf-like repeats in one or both binding ligands contribute to the binding site. Since binding to fibrillin-2 was demonstrated with rF23, only two cb EGf-like repeats in fibrillin-1 (nos. 1 and 2) could be responsible for the active binding site. In fibrillin-2, the binding site has not been narrowed down. Therefore, all of the nine cb EGf-like repeats in human fibrillin-2 are possible candidate domains for the binding site.

A Marfan mutation has been identified in most of the 43 cb EGf-like repeats present in fibrillin-1 (Dietz and Pyeritz, 1995). However, to date, mutations have not been found to occur in cb EGf-like repeats nos. 1 and 2. If these repeats contribute to the binding site for fibrillin-2, these data might again indicate that binding of fibrillin-2 to fibrillin-1 is important to human development. Mutations in these domains might lead to embryonic death. In the two fibrillins, cb EGf-like repeats nos. 1 and 2 are practically identical at the level of primary structure.

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Therefore, it is possible that fibrillin-2 may also bind fibulin-2. This interaction may be important particularly during early embryogenesis when fibrillin-2 is more highly expressed than fibrillin-1 (Zhang et al., 1995b).

Acknowledgments—We thank J. Jay Gambee, Jeffrey Bonder, Catherine Ridgway, and Robert Ono for excellent technical assistance. Bio-tiling of fibrillin-2 was kindly performed by Dr. Gunter Kostka.

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