Homotypic Fibrillin-1 Interactions in Microfibril Assembly*

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We have defined the homotypic interactions of fibrillin-1 to obtain new insights into microfibril assembly. Dose-dependent saturable high affinity binding was demonstrated between N-terminal fragments, between furin processed C-terminal fragments, and between these N- and C-terminal fragments. The N terminus also interacted with a downstream fragment. A post-furin cleavage site C-terminal sequence also interacted with the N terminus, with itself and with the furin-processed fragment. No other homotypic fibrillin-1 interactions were detected. Some terminal homotypic interactions were inhibited by other terminal sequences, and were strongly calcium-dependent. Treatment of an N-terminal fragment with N-ethylmaleimide reduced homotypic binding. Microfibril-associated glycoprotein-1 inhibited N- to C-terminal interactions but not homotypic N-terminal interactions. These fibrillin-1 interactions are likely to regulate pericellular fibrillin-1 microfibril assembly.

Fibrillins are large multidomain glycoproteins (~340 kDa) and the major structural components of a class of 10–12-nm extracellular matrix microfibrils that are widely distributed in connective tissues (1–3). In elastic tissues such as aorta, lung, and skin, they are associated with tropoelastin deposition during elastic fibrillogenesis, and form an outer mantle for mature elastic fibers (4, 5). Microfibril arrays are also abundant in dynamic tissues that do not express elastin, such as the cilary zonules of the eye (6). Structural analyses of isolated fibrillin-rich microfibrils have revealed a complex 56-nm “beads-on-a-string” appearance, whereas the predicted length of a fibrillin monomer is ~160 nm (7–10). Mutations in fibrillin-1 cause Marfan syndrome, a heritable disease associated with severe aortic, ocular, and skeletal defects because of defective elastic fibers (11). There are three closely related fibrillin isoforms that have distinct, but overlapping, developmental, and adult tissue distributions (12–14). Fibrillin-1 contains 47 epidermal growth factor (EGF)-1-like domains, 43 of which are calcium binding (chEGF)-like domains, seven 8-cysteine (TB) modules, two hybrid motifs with similarities to both chEGF-like domains and TB motifs, and a proline-rich region that may act as a hinge region (12). In fibrillin-2, this sequence is glycine-rich (13), and in fibrillin-3 it is proline/glycine-rich (14). The linkage to Marfan syndrome, and its abundance in developing and adult tissues (11, 15), confirm fibrillin-1 as the major fibrillin isoform in elastic fibers. C-terminal furin processing of fibrillin-1 is important for extracellular fibrillin-1 deposition (16, 17). However, mass spectrometry analysis of isolated tissue microfibrils has shown that at least some unprocessed molecules are present in tissue microfibrils.2

The molecular mechanisms by which fibrillins assemble into mature microfibrils remain unresolved. Several models of fibrillin alignment in microfibrils have been proposed (19–21). Head-to-tail alignment of fibrillin-1 molecules within microfibrils has been proposed on the basis of antibody localizations (19, 20). Complex intramolecular folding in 56-nm microfibrils was also demonstrated by interbead antibody epitope reversal (20). A one-third staggered arrangement has also been suggested (21). Lateral fibrillin-1 assembly is another critical, but unexplained, feature of microfibril assembly. Electron microscopy of isolated microfibrils, and mass mapping, suggest that there are eight molecules in cross-section (20, 22, 23). Recombinantly expressed N-terminal regions of fibrillin-1 have a tendency to dimerize (24, 25), whereas the N-terminal half of the molecule interacts with the C-terminal half (26). An unpaired cysteine residue in the first hybrid domain may covalently link aligned fibrillin-1 molecules (27). In vitro studies of interactions between fibrillin-1 and other elastic fiber molecules have revealed that its N terminus is highly interactive, binding to microfibril-associated glycoprotein-1 (MAGP-1) (28, 29) and fibrillin-2 (30). It is unclear whether any of these interactions are mutually exclusive.

We have undertaken a detailed analysis of fibrillin-1 homotypic interactions, using recombinant fragments that span the entire coding region of human fibrillin-1, to define fibrillin-1 sequences that interact. The N terminus binds very strongly to itself and to an overlapping downstream sequence. The furin-processed C terminus and the proteolytically released C-terminal 20-kDa fragment both bind homotypically and tightly to the N terminus. No other homotypic fibrillin-1 interactions were detected. MAGP-1 inhibits the N- to C-terminal interaction but not the N- to N-terminal interaction. These interactions may regulate linear head-to-tail and lateral fibrillin-1 assembly.
EXPERIMENTAL PROCEDURES

Recombinant Fibrillin-1 Fragments and Full-length MAGP-1—Recombinant human fibrillin-1 fragments PF1 encoded by exons 1–11 (residues 1–489), PF2 encoded by exons 9–17 (residues 330–722), PF3 encoded by exons 1–17 (residues 1–722), PF4 encoded by exons 1–8 (residues 1–329), PF5 encoded by exons 18–25 (residues 723–1069), PF7 encoded by exons 24–30 (residues 952–1279), PF8 encoded by exons 30–38 (residues 1238–1605), PF9 encoded by exons 37–43 (residues 1528–1656), PF10 encoded by exons 41–52 (residues 1688–2165), PF11 encoded by exons 35–52 (residues 2055–2443), and PF12 encoded by exons 57–65 (residues 2402–2871) (12) were expressed using the pGEX4T-3 mammalian expression system in E. coli. PF1, PF2, PF3, PF5, PF7, PF8, PF9, PF10, PF11, and PF12 fragments were N-glycosylated as predicted from the primary sequence. PF13 was furin processed on secretion that removed an N-glycosylated C-terminal post-furin cleavage fragment (Fig. 1B). For some experiments, PF13 was treated with the serine protease trypsin (10 units per mg). In other experiments, the addition of decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (Sigma) in TBS/CaCl2 at room temperature for 10–15 min, and read at a wavelength of 405 nm. For both methods, wells were washed four times and the color developed using 40 mM 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution. (Sigma) for 10–20 min at room temperature. Plates were read at a wavelength of 405 nm. All assays were performed in triplicate and repeated at least twice to confirm results.

Calcium Dependence Binding Assays—Binding assays were performed as above, except that dilution buffers and washes were with or without calcium (0.001 M CaCl2). The wash buffer contained 1 mg/ml heat-denatured BSA in Tris-buffered saline (TBS). Solid-phase protein fragments at 0.12 μM were incubated for 1 h at 4 °C in the presence or absence of 10 mM EDTA, before adherence to the plate and overnight incubation at 37 °C. The wells were blocked with 10 mg/ml filtered, heat-denatured BSA for 3 h, and washed with wash buffer with or without calcium, depending on whether or not the solid phase had been treated with EDTA. Each soluble ligand was biotinylated and dialyzed, as described above. Each protein ligand was incubated at 4 °C in the presence or absence of 10 mM EDTA for 1 h, re-equilibrated in TBS with or without calcium, then added to the wells at (0.02 μM) and incubated overnight at 37 °C. Plates were washed three times with wash buffer with or without calcium, before detection of bound fibrillin-1. Biotinylated ligand was quantified by incubating fibrillin-1 fusion protein of ExtraAvidin peroxidase conjugate (Sigma) in TBS with or without CaCl2, for 10–20 min at room temperature. Wells were washed four times with wash buffer with or without calcium, and the color was developed following addition of 40 μM ABTS solution for 10–20 min at room temperature. Plates were read at a wavelength of 405 nm. All experiments were done in triplicate wells, and repeated at least twice to confirm results.

Fibrillin-1 Inhibition Binding Assays—Inhibition binding assays were also conducted using both non-biotinylated and biotinylated soluble protein ligands. Flat bottomed microtiter plates were coated with N-terminal (PF4) or C-terminal (PF13) fragments at 0.12 μM in TBS/CaCl2 overnight at 37 °C. Non-specific binding sites were blocked with TBS/CaCl2 containing 10 mg/ml BSA, at room temperature for at least 3 h. The plates were washed with wash buffer (3 × 200 μl) and incubated with 0.02 μM (or as specified) of the second non-biotinylated soluble ligand, prior to solid-phase binding assay with TBS/CaCl2 overnight at 37 °C. The plates were washed again three times, then 0.02 μM (or as specified) of the second biotinylated protein in TBS/CaCl2 was added, overnight at 37 °C. Control wells were incubated overnight in TBS/CaCl2 with the first non-biotinylated soluble ligand omitted, prior to addition of the biotinylated second soluble ligand. After a further three washes, plates were incubated with 1,000 dilution of biotinylated Anti-EGF (Sigma) and washed twice to confirm results.

MAGP-1 Inhibition Binding Assays—Assays were performed as described above for competition assays involving only fibrillin-1 protein fragments. Flat bottomed microtiter plates were coated with N-terminal (PF4) or C-terminal (PF13) fragments at 0.12 μM in TBS/CaCl2 overnight at 37 °C. Non-specific binding sites were blocked as described. The plates were washed with wash buffer (3 × 200 μl) and incubated with increasing concentrations of non-biotinylated MAGP-1 in TBS/CaCl2 overnight at 37 °C. The plates were then washed with wash buffer (3 × 200 μl) and incubated with 0.03 μM biotinylated N- (PF4) or C- (PF13, CP) terminal fibrillin-1 protein fragments in TBS/CaCl2 overnight at 37 °C. The reaction was completed as described above.

N-Ethylmaleimide Treatment—Solid-phase assays were performed, as described above, to investigate N-Ethylmaleimide (NEM) capping of the free cysteine within the PF4 hybrid domain (27). PF4, at a concentration of 0.12 μM was preincubated at 4 °C with or without 2 μM NEM (molar excess) for 1 h. NEM-treated PF4 was allowed to adsorb to flat-bottomed microtiter plates, prior to solid-phase binding assays with soluble untreated PF4, as outlined above.

Dissociation Constants for Fibrillin-1 Interactions—We previously used surface plasmon resonance with a BIAcore biosensor (BIAcore 3000, BIAcore, Uppsala, Sweden) to screen full-length recombinant human fibrillin-1 fragments that together span the full-length molecule (residues 1–2166) for interactions with MAGP-1 and tropoelastin (29). In those experiments, MAGP-1 or tropoelastin was attached to CM5 chips, with fibrillin-1 fragments as soluble analytes. Here it was possible to bind the C-terminal fragment CP to CM5 BIAcore chips for analysis of this interaction with PF4. However, fibrillin-1 PF1, PF3, PF4, and PF13 fragments were found not to attach stably to either CM5 or streptavidin-coated chips, in contrast to a previous report of N- and...
C-terminal fibrillin-1 halves (26). Therefore, we derived kinetic data on fibrillin-1-fibrillin-1 interactions using solid-phase assays, percentage saturation was analyzed using GraphPad Prism software and Scatchard plots.

RESULTS

Localization of Homotypic Fibrillin-1 Interaction Sites

We investigated fibrillin-1 homotypic interactions to gain new insights into how microfibrils assemble. To date, there has not been a comprehensive analysis of fibrillin-1 interactions, although it was reported that the N-terminal half of fibrillin-1 (encoded by exons 1–36) interacted with the C-terminal half of the molecule (encoded by exons 36–65) (26). We have used shorter overlapping human fibrillin-1 fragments encompassing the entire sequence (Fig. 1A), and full-length human MAGP-1 in solid-phase binding and inhibition assays, and BIAcore analyses. The interactions show dose dependence and saturation kinetics.
Fibrillin-1 N-terminal Interactions—Homotypic N-terminal fibrillin-1 interactions were studied using solid-phase binding assays. Immobilized PF4 (encoded by exons 1–8) interacted strongly with soluble PF4 (Figs. 2A and 3A), so a homotypic interaction site must be present within this N-terminal fragment. PF4 also interacted strongly with downstream fragment PF2 (encoded by exons 9–17) (Fig. 2A), but PF2 bound only weakly to itself (not shown). The longer N-terminal fragment PF1 also bound PF2 (not shown). Thus, N-terminal sequences can interact with PF2. This interaction could stabilize predicted folding at the proline-rich region.

Fibrillin-1 N-terminal Interactions with Central Domains—Using solid-phase assays, the ability of PF1 and PF2 to interact with central domains was investigated to determine whether there are any strong interactions that might be important in microfibril assembly or packing. Screening of fragments PF5, PF7, PF8, PF9, PF10, and PF12 (Fig. 1) showed no significant binding to PF1 or PF2, or to each other (not shown).

Fibrillin-1 N- and C-terminal Interactions—We next investigated the ability of N- (PF4) and C-terminal (processed PF13) domains to interact, using solid-phase binding assays (Fig. 3, C and D). Such an interaction could form the basis for head-to-tail linear fibrillin-1 assembly (19, 20). Immobilized N-terminal PF4 strongly bound soluble C-terminal PF13 and, similarly, immobilized PF13 strongly bound to soluble PF4 (Fig. 3, C and D). Thus, there is a major N- to C-terminal interaction between PF4 and the C-terminal sequence encoded by exon 57 to the furin cleavage site.

The interaction between PF4 and PF13 was dependent on which fragment was immobilized on the wells (Fig. 3, C and D). The interaction between immobilized PF4 to soluble PF13 occurred more rapidly and strongly than that between immobilized PF13 to soluble PF4. When PF13 was immobilized, the ability to interact with the N terminus may have been limited.
Fibrillin-1 Interactions in Assembly

Fig. 3. Non-linear regression analysis of N- and C-terminal fibrillin-1. Fibrillin-1 fragments were coated to the plastic surfaces of multiwell plates, and then incubated with soluble biotinylated fibrillin fragments at increasing concentrations. Soluble fragments that bound to immobilized fibrillin-1 fragments were quantified by incubating with ExtrAvidin peroxidase, then color development using ABTS solution and detection at 405 nm. Nonspecific binding was subtracted. Results are shown as the mean ± S.E. of triplicate values. Saturation binding curves are shown, with calculated maximum response values for each concentration, plotted against concentration. Using non-linear regression, \( K_D \) and \( B_{max} \) values were also calculated using GraphPad Prism version 2.0 (see Table I). A, the fibrillin-1 N-terminal fragment PF4 was immobilized, then incubated with soluble biotinylated PF4. B, the fibrillin-1 C-terminal fragment PF13 was immobilized, then incubated with soluble biotinylated PF13. C, the fibrillin-1 N-terminal fragment PF4 was immobilized, then incubated with soluble biotinylated PF13. D, the fibrillin-1 C-terminal fragment PF13 was immobilized, then incubated with soluble biotinylated PF4.

because of conformational effects or masking of an interactive site. Immobilized PF2 did not bind PF13 (not shown).

The post-furin cleavage site fragment (CP) also interacted strongly with PF4 (Fig. 2, B and C). Thus, a second N-terminal binding site is present within this processed C-terminal fragment.

Fibrillin-1 C-terminal Interactions—The post-furin cleavage site CP fragment was self-associated (Fig. 2B) and interacted with PF13 (Fig. 2D). Immobilized PF13 also bound strongly to itself (Fig. 3B).

Characterization of Interactions

The molecular nature of the N- to N-, N- to C-, and C- to C-terminal interactions was investigated with respect to binding affinities, inhibition assays, calcium dependence, the contribution of a free cysteine residue within the first hybrid motif, and the influence of MAGP-1 binding to the N terminus.

Dissociation Constants for N-N, N-C, and C-C Fibrillin-1 Interactions—Dissociation constants were determined for the interactions between PF4-PF4, PF4-PF13, and PF13-PF13, using solid-phase binding assays, percentage saturation, and Scatchard plots (Fig. 3, A–D; Table I, solid-phase assays), and for CP-PF4 using BIAcore analysis (Fig. 4; Table I, BIAcore analyses).

The interactions between fibrillin-1 terminal sequences were all strong binding events, with \( K_D \) values in the low nanomolar range. The strongest interaction was between N-terminal PF4 and C-terminal PF13, with PF4 as the immobilized ligand. By one-site binding nonlinear regression analysis, this interaction had a dissociation constant (\( K_D \)) of 5.5 ± 0.5 nm. When PF13 was immobilized and PF4 was the soluble ligand, the \( K_D \) was 11.1 ± 1.5 nm. The PF4 to PF4 N-terminal interaction had a \( K_D \) of 11.6 ± 1.0 nm. The PF13 to PF13 C-terminal interaction had a \( K_D \) of 14.2 ± 1.5 nm. Dissociation constants determined by Scatchard plot analysis gave \( K_D \) values in a similar nanomolar range. BIAcore analysis identified the highest affinity interaction to be between PF4 and CP, with a \( K_D \) of 3.3 nm.

Inhibition Binding Assays—Inhibition binding assays were performed to investigate whether N- and C-terminal binding sites are mutually exclusive (Fig. 5). The N-terminal interactions were challenged with increasing amounts of C-terminal PF13. Soluble PF13, then PF4 were sequentially added to the wells, with appropriate washing and blocking steps. When the molar ratio of soluble PF13 to immobilized and soluble PF4 was 0.7:1, addition of soluble 70% processed PF13 partially inhibited (by ~20%) subsequent soluble PF4 binding to immobilized PF4. At the molar ratio of 2:1, the PF4-PF4 interaction was strongly inhibited by PF13 (by ~80%). This result implies that both PF13 and PF4 bind to PF4 at the same, or an overlapping site. In contrast, PF4 did not interfere with the PF13-PF13 interaction. At the molar ratio for soluble PF4 to immobilized and soluble PF13 of 1:0.7, there was no inhibition of the PF13-PF13 interaction. Thus, PF4 and PF13 bind to PF13 at separate sites. Preincubation of soluble PF13 with CP also inhibited the PF4-PF4 interaction up to 90% (not shown).

Calcium Dependence of N- and C-terminal Interactions—The calcium dependence of fibrillin-1 interactions was determined following EDTA treatment and subsequent steps in calcium-free buffers (Fig. 6). For the N-terminal homotypic interactions (PF4-PF4 and PF1-PF1), removal of calcium had virtually no effect on binding (Fig. 6; not shown). Thus, the two cbEGF-like

### Table I

| Solid-phase assays | \( K_D \) S.E. | Scatchard \( K_D \) |
|-------------------|--------------|--------------------|
| PF4-PF4           | 11.6 ± 1.1   | 21.4 ± 2.3         |
| PF4-PF13          | 5.5 ± 0.52   | 14.2 ± 1.2         |
| PF13-PF13         | 14.2 ± 1.5   | 15.5 ± 0.9         |
| PF13-PF4          | 11.1 ± 1.5   | 30.2 ± 7.9         |

**BIAcore analyses**

| \( K_D \) | \( 10^7 \text{ M}^{-1} \text{s}^{-1} \) | \( K_D \) |
|-----------|----------------------------------------|--------|
| CP-PF4    | 0.667 ± 0.148                          | 3.33 ± 1.45 |

Calcium Dependence of N- and C-terminal Interactions—The calcium dependence of fibrillin-1 interactions was determined following EDTA treatment and subsequent steps in calcium-free buffers (Fig. 6). For the N-terminal homotypic interactions (PF4-PF4 and PF1-PF1), removal of calcium had virtually no effect on binding (Fig. 6; not shown). Thus, the two cbEGF-like
domains within PF1 and PF4 (encoded by exons 7 and 8) are probably not directly involved in these N-terminal interactions. The interaction between PF1 and PF2 was also unaffected by removal of calcium (not shown) so the four PF2 cbEGF-like domains (encoded by exons 12–15) are unlikely to be involved in this interaction.

Effects of EDTA treatment were then determined for the PF4-PF13 interaction (Fig. 6). With PF4 immobilized, removal of calcium reduced PF13 binding by up to 70%. With PF13 immobilized, removal of calcium reduced this interaction by up to 40% (not shown). EDTA treatment also had a profound disruptive effect on C-terminal (PF13-PF13) homotypic interactions (Fig. 6). With both immobilized and soluble PF13 EDTA treated, binding was reduced by 90%. Thus, the ability of PF13 to interact both homotypically and with the N terminus is strongly calcium dependent, so key C-terminal interactive sequences are within the array of seven cbEGF-like domains encoded by exons 58–63.

Effects of NEM on N-terminal Fibrillin-1 Interactions—A cysteine residue with a free thiol group that is surface accessible has previously been identified within the first hybrid domain encoded by exon 6 that contains nine cysteines (27). We studied homotypic N-terminal fibrillin-1 interactions, using the PF4 fragment, in the presence or absence of NEM that caps free cysteines. After NEM treatment, total PF4-PF4 binding was reduced by 50% and saturation occurred at a lower concentration of soluble ligand, although binding affinity was unaffected (Fig. 7). This experiment implicates the first hybrid motif, indirectly or directly, in this interaction.

Effects of MAGP-1 Binding on Fibrillin-1 N-terminal Interactions—Several other molecules are known to associate with tissue microfibrils (28–30). We investigated the possibility that binding of one of the major microfibril-associated molecules, MAGP-1, which interacts with the N terminus of fibrillin-1 (28, 29), may influence N- to N-terminal (PF4-PF4) or N- to C-terminal (PF4-PF13) interactions, using inhibition binding assays. MAGP-1 at saturating PF4 binding concentrations (29) did not inhibit the PF4-PF4 interaction, but did inhibit the PF4-PF13 interaction by ~30% (not shown). BIAcore analysis of the ability of MAGP-1 to inhibit the CP to PF4 interaction showed 50% inhibition at 0.04 μM MAGP-1, increasing to 100% inhibition at 1.2 μM MAGP-1 (Fig. 8). Thus, MAGP-1 association with the N terminus has the potential to influence linear N- to C-terminal fibrillin-1 assembly.

DISCUSSION

Previous immunoelectron microscopy analyses of isolated fibrillin-rich microfibrils have indicated that fibrillin-1 molecules adopt a head-to-tail arrangement within assembled microfibrils (19, 20). In this study, we have undertaken a detailed examination of homotypic fibrillin-1 interactions to shed new light on the molecular basis of fibrillin-1 assembly. A previous study showed that N- and C-terminal halves of fibrillin-1 can interact (26). Here, we have shown that fibrillin-1 homotypic interactions only involve the terminal sequences, and we have localized specific regions of the N and C termini that both self-associate and interact with each other with high affinity. The N- to N-terminal interaction is calcium-independent, whereas the furin-processed C-terminal (PF13) interactions with the N terminus and with itself are strongly calcium-dependent. The post-furin C-terminal cleavage site sequence (CP) also interacts strongly with the N terminus, with itself, and with the furin-processed C-terminal region. These interactions may regulate microfibril assembly (Fig. 9).

The specificity and nature of the N- and C-terminal interactions we have identified suggest that they play a crucial role in linear and lateral assembly. Because the N-terminal sequence encoded by exons 1–8 (PF4) interacts strongly with itself and contains a free cysteine (encoded by exon 6) (27) is implicated, indirectly or directly. MAGP-1 binds the sequence encoded by exons 1–3 (28) but does not inhibit N- to N-terminal binding, and we also have data showing that a Marfan syndrome mutation in exon 2 ablates MAGP-1 binding3; thus, the N-terminal homotypic binding site is probably within exons 3–6 (Fig. 9, A and D). N-terminal (PF4) interactions with the downstream non-overlapping fibrillin-1 fragment PF2 (encoded by exons 9–17) may be facilitated by proline-rich region folding, and may serve to stabilize such a folded arrangement. Because the N-PF2 interaction is calcium-independent (not shown), it may be mediated by sequences encoded by exons 1–6 (within PF4) and either 9–11 or 16–17 (within PF2), respectively (Fig. 9, A, B, and D). However, we predict that the PF2 interaction site is most likely to be within TB2 encoded by exons 16/17 because PF1-PF2 and PF4-PF2 interactions were similar.

Because C-terminal PF13 strongly inhibits PF4-PF4 binding, there may be a single or overlapping N-terminal site for binding these N- and C-terminal sequences (Fig. 9, A, C, and F). Interestingly, MAGP-1 binding to the N terminus inhibits the association of the N and C termini (PF13 to ~30%; CP to >90%), indicating that these C-terminal sequences bind within the sequence encoded by exons 1–3. However, a Marfan disease-causing mutation in the domain encoded by exon 2 did not disrupt either N- to N-, or N- to C-terminal binding (data not shown). Thus, the PF4-PF13 interaction could be in the domain encoded by exon 3, which overlaps with the N- to N-terminal interactive region (domains encoded by exons 3–6). This N-terminal sequence has a net positive charge, whereas the last five C-terminal cbEGF-like domains (encoded by exons 60–63)

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have a net negative charge even with bound calcium, so electrostatic interactions may play some role in these N- to C-terminal interactions.

Self-association of the furin-processed C-terminal fragment PF13, and its interactions with the N terminus are strongly calcium-dependent, so these interactions must involve the cbEGF-like domains encoded by exons 58–63 (Fig. 9, C and E). Because PF4 does not inhibit PF13-PF13 interactions, there must be distinct C-terminal sites within PF13 for N- to C- and C- to C-terminal interactions. Bound biotinylated second ligand was quantified using the ABTS colorimetric assay, with plates read at 405 nm. All experiments were done in triplicate wells, and repeated at least twice. Black bars are 0.7:1.0 M, PF13:PF4. Open white bar is 2.0:1.0 M, PF13:PF4. Results are shown as the mean ± S.E. of the triplicate values.

FIG. 6. Calcium dependence of fibrillin-1 interactions. PF4 interactions were not calcium-dependent but PF13 interactions were calcium dependent. Binding assays in the absence of calcium were performed as outlined under "Experimental Procedures." Briefly, solid-phase protein fragments for immobilization were preincubated in the presence of 10 mM EDTA. Dilution buffers and washes were without calcium (0.001 M CaCl₂). Soluble ligands were biotinylated, treated with EDTA, and dialyzed into dilution buffer. Bound biotinylated ligand was quantified using the ABTS colorimetric assay, with plates read at 405 nm. All experiments were done in triplicate wells, and repeated at least twice. Results are shown as the mean ± S.E. of triplicate values. NE refers to EDTA-treated PF4. CE refers to EDTA-treated PF13. The black bar is the NE-NE interaction; the hatched bar is the NE-CE interaction; the open white bar is the CE-CE interaction.

The fibrillin-1 N terminus is highly interactive and has been shown, in vitro, to associate with molecules such as MAGP-1 (28, 29) and fibulin-2 (30). It is of interest to determine whether the N- to N- and N- to C-terminal fibrillin-1 interactions identified here, and interactions with associated molecules are mutually exclusive. We have shown here that binding of MAGP-1 to the fibrillin-1 N terminus inhibits N- and C- (PF13 and CP) terminal interactions. If, in vivo, fibrillin-1 molecules associate with MAGP-1 before assembly, then MAGP-1 could profoundly influence N- to C-terminal interactions and head-to-tail linear assembly. The expression of MAGP-1 and fibrillin-1 are known to overlap in developing tissues (18). This is the first demonstration that MAGP-1 may influence linear fibrillin-1 assembly.

FIG. 7. Effect of NEM on homotypic PF4-PF4 interaction. N-terminal (PF4) fibrillin-1 interactions were examined in the presence or absence of NEM, which caps free cysteines. PF4 was preincubated with or without 2 mM NEM (molar excess) for 1 h. NEM-treated PF4 was then adsorbed to flat-bottomed microtiter plates, prior to solid-phase binding assays with soluble biotinylated untreated PF4. Bound biotinylated ligand was quantified using the ABTS colorimetric assay, with plates read at 405 nm. All experiments were done in triplicate wells, and repeated at least twice. NEM treatment reduced total PF4-PF4 binding by 50% and saturation occurred at a lower concentration of soluble ligand. ■, minus NEM; ▲, plus 2 mM NEM. All experiments were done in triplicate wells, and repeated at least twice. Results are shown as the mean ± S.E. of the triplicate values.

FIG. 8. MAGP-1 effects on N- and C-terminal fibrillin-1 interactions. BIAcore analysis of the ability of MAGP-1 to inhibit the CP to PF4 interaction showed at 50% inhibition at 0.04 μM MAGP-1, increasing to 100% inhibition at 1.2 μM MAGP-1. MAGP-1 association with the N terminus has the potential to influence linear N- to C-terminal fibrillin-1 assembly.
The role of C-terminal furin processing in fibrillin-1 assembly remains unclear. It has been shown that mutations that disrupt processing inhibit fibrillin-1 deposition in the extracellular matrix and that processing is necessary for extracellular fibrillin-1 deposition matrix (16, 17). However, the functional importance of this extreme C-terminal sequence is also highlighted by the mutations here that cause severe Marfan syndrome (Marfan syndrome mutation database umd.necker.fr). Moreover, we have detected some post-furin cleavage site tryptic peptides in zonular and skin microfibrils using mass spectrometry. The current study shows that CP interacts strongly with the N terminus, and also with processed C-terminal sequence and homotypically, suggesting that it plays an essential role in aligning fibrillin-1 molecules during early assembly prior to processing. This cleaved sequence may possibly be retained within the periodic bead-like structures characteristically observed by electron microscopy of isolated microfibrils (20).

In summary, the data provide new information on how fibrillin-1 molecules assemble. Lateral alignment could be facilitated by N- to N- and/or C- to C-terminal interactions. Head-to-tail assembly could be driven by N- to C-terminal interactions. These specific interactions are likely to be dominant factors in microfibril assembly, and imply that fibrillin-1 is inherently capable of self-assembly. However, other molecules may modulate assembly. In this context, it is interesting that MAGP-1 can inhibit N- to C-terminal interactions. MAGP-1 has been implicated as a link between fibrillin-1 and tropoelastin, but it may also function to regulate aspects of microfibril assembly.

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