Molecular Basis of Elastic Fiber Formation

CRITICAL INTERACTIONS AND A TROPOELASTIN-FIBRILLIN-1 CROSS-LINK*

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We have investigated the molecular basis of elastic fiber formation on fibrillin microfibrils. Binding assays revealed high affinity calcium-independent binding of two overlapping fibrillin-1 fragments (encoded by central exons 18–25 and 24–30) to tropoelastin, which, in microfibrils, map to an exposed “arms” feature adjacent to the beads. A further binding site within an adjacent fragment (encoded by exons 9–17) was within an eight-cysteine motif designated TB2 (encoded by exons 16 and 17). Binding to TB2 was ablated by the presence of N-terminal domains (encoded by exons 1–8) and reduced after deleting the proline-rich region. A novel transglutaminase cross-link between tropoelastin and fibrillin-1 fragment (encoded by exons 9–17) was localized by mass spectrometry to a sequence encoded by exon 17. The high affinity binding and cross-linking of tropoelastin to a central fibrillin-1 sequence confirm that this association is fundamental to elastic fiber formation. Microfibril-associated glycoprotein-1 showed calcium-dependent binding of moderate affinity to fibrillin-1 N-terminal fragment (encoded by exons 1–8), which localize to the beads. Microfibril-associated glycoprotein-1 thus contributes to microfibril organization but may also form secondary interactions with adjacent microfibril-bound tropoelastin.

Elastic fibers are one of the major insoluble fiber systems of connective tissues, providing elasticity and resilience to elastic tissues such as blood vessels, lungs, skin, and ligaments (1, 2). They morphologically comprise an elastin core surrounded by a mantle of fibrillin-rich microfibrils, and their distinct tissue-specific arrangements reflect different biomechanical requirements. Elastic fiber formation in the extracellular space is a complex developmentally regulated process that has been visualized by electron microscopy studies as the accretion of tropoelastin on preformed bundles of fibrillin microfibrils in the pericellular space. The microfibril template thus profoundly influences tropoelastin deposition and the organization of mature elastic fibers as well as their biomechanical properties (3).

Understanding of elastic fiber formation requires delineation of the molecular basis of the critical early stage of tropoelastin deposition on microfibrils.

Fibrillin-1 is a large cysteine-rich multidomain glycoprotein that polymerizes in the extracellular space in a head-to-tail manner to form microfibrils that provide a force-bearing structural framework for dynamic connective tissues (2, 4, 5). It contains 47 epidermal growth factor (EGF)-like domains: 43 calcium-binding epidermal growth factor (cEGF)-like domains, seven 8-cysteine (TB) modules, two hybrid motifs, and a proline-rich region that may act as a hinge region. Mutations in fibrillin-1 cause Marfan syndrome, a heritable disease associated with severe aortic, ocular, and skeletal defects due to defective elastic fibers (6). This linkage to Marfan syndrome and its developmental distribution (7) confirm fibrillin-1 as the major fibrillin isoform in elastic fibers.

In addition to fibrillin-1, other microfibril-associated molecules have been identified (2). The best candidate for an integral structural component is microfibril-associated glycoprotein-1 (MAGP-1), which was first identified as a microfibril-associated molecule in reductive denaturing tissue microfibril extracts (8) and which routinely co-localizes with microfibrils in elastic and nonelastic tissues (2, 9). MAGP-1 was shown to bind to an N-terminal fibrillin-1 peptide (10), to tropoelastin (11), and to extracellular matrix through interactions of its C-terminal matrix binding domain (12). An antibody inhibition study suggested that the interaction between tropoelastin and microfibrils might be mediated by the N-terminal half of MAGP-1 (13). Tropoelastin was shown to bind a fibrillin-1 fragment (encoded by exons 10–17) through interactions involving its lysine side chains (14). Ternary complexes of MAGP-1, fibrillin, and decorin and of MAGP-1, tropoelastin, and biglycan have been identified in vitro (15, 16). Whereas small leucine-rich proteoglycans may contribute to elastic fiber formation, available evidence indicates that the key molecules in this process are tropoelastin, fibrillin-1, and MAGP-1.

The molecular basis of how tropoelastin binds microfibrils during elastic fiber formation remains unclear, and no comprehensive screening of interactions with fibrillin-1 has been reported. To resolve this critical issue, we have defined the interactions between tropoelastin and MAGP-1 with fibrillin-1 and their binding affinities. We have also identified and localized a novel specific transglutaminase cross-link between tro-
poelastic and fibrillin-1. The data confirm that tropoelastin strongly binds and is cross-linked to a central fibrillin-1 sequence that is exposed on microfibrils, whereas MAGP-1 binds N-terminal fibrillin-1 and contributes to the microfibril template. These data thus provide essential new insights into the molecular basis of elastic fiber formation.

**EXPERIMENTAL PROCEDURES**

**Recombinant Fibrillin-1 Fragments**—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 19–26 (residues 723–2166)), PF6 (encoded by exons 23–25 (residues 910–1069)), PF7 (encoded by exons 24–30 (residues 925–1279)), PF8 (encoded by exons 30–38 (residues 1283–1605)), PF9 (encoded by exons 37–43 (residues 1528–2166)), PF10 (encoded by exons 41–52 (residues 1688–2165)), PF11 (encoded by exons 37–52 (residues 1528–2165)), PF12 (encoded by exons 50–58 (residues 2055–2443)), and PF13 (encoded by exons 58–65 (residues 2402–2871)) (17) were expressed and purified in milligram amounts using a mammalian terminal His6 tag following the signal peptide to allow rapid fragment purification. Residues 1528–1605 of PF12 were N-glycosylated as predicted from the primary sequence; PF13 was furin-processed, which removed an N-glycosylated C-terminal fragment. Fragments PF1–PF8, PF12, and PF13 are described in this paper for the first time. Fragments PF9, PF10, and PF11 have previously been described (22). Fibrillin-1 fragments expressed in a similar system are correctly folded (23, 24, 25). Production of recombinant human tropoelastin used in this study has been described (26, 27).

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**Fibrillin-1 Interactions with Tropoelastin and MAGP-1**

Fibrillin-1 binding assays were conducted using both nonbiotinylated and biotinylated soluble proteins. The colorimetric assay described above, using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (Sigma) for 10–20 min at room temperature. Plates were read at a wavelength of 405 nm. Any non-specifically bound fibrillin-1 or fibrillin-1 binding was detected by blocking precoated wells before incubation with biotinylated ligand. For the antibody detection assay, blocking, binding, and detection of binding was done using infected insect cell extract and antibody only. All assays were performed in triplicate and repeated at least twice to confirm observed results. We used biotinylated elastin as it is a widely used and sensitive molecular detection system. In all cases, similar patterns of binding were recorded, so biotinylation did not affect the binding interactions.

**Competition Binding Assays**—Competition binding assays were also conducted using both nonbiotinylated and biotinylated soluble proteins. The colorimetric assay described above, using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (Sigma) for 10–20 min at room temperature. Plates were read at a wavelength of 405 nm. All experiments were performed in triplicate of 10–400 nM.

**Biacore 3000 Kinetic Analysis of Molecular Interactions**—For kinetic binding studies of MAGP-1 and fibrillin-1 fragments by surface plasmon resonance, a Biacore biosensor unit was used (Biacore 3000; Biacore AB). MAGP-1 or tropoelastin was immobilized on CM5 sensor chips by amine coupling using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl and N-hydroxysuccinimide, and NHS-activated dextran matrix as described by the manufacturer. The instrument was operated in a low reflecting mode with a flow rate of 30 ml/min. MAGP-1 binding analysis was performed for fibrillin-1 ligands binding to MAGP-1 and full-length MAGP-1 and was rotared at room temperature for 30 min with an approximate 10-fold molar excess of 10 mg/ml solution of Immunosure sulfon-N-hydroxysuccinimide ester-biotin (Pierce) diluted in PBS. Each mixture was then dialyzed against several changes of 0.02 M Tris/HCl, pH 7.4, containing 0.1 mM NaCl and 0.001 mM CaCl2 (TBSa/CaCl2) to remove excess biotin. The plates were washed with 3 ml of TBSa/CaCl2, overnight at 37 °C. The plates were then washed with 3 ml of TBSa/CaCl2, 5% BSA at least 2 h at room temperature to block nonspecific binding sites, followed by three washes with TBSa/CaCl2 containing 0.1% BSA. Initially, 5 μg/ml fibrillin-1 protein fragments were column-purified (Fig. 1) and these were designated PF2 and PF3 that lacked the proline-rich region were also produced for PF2 and PF3; these were then blocked prior to the addition of soluble fibrillin-1 or tropoelastin. The data confirm that tropoelastin strongly binds and is cross-linked to a central fibrillin-1 sequence that is exposed on microfibrils, whereas MAGP-1 binds N-terminal fibrillin-1 and contributes to the microfibril template. These data thus provide essential new insights into the molecular basis of elastic fiber formation.
mM NaCl for 60 s. The chip was then stabilized for 20 min using HBS-Ca before the next injection was carried out. The analyte was simultaneously passed over a blank capped flow cell, and this baseline was subtracted from the experimental flow cell. The maximum relative response value for each injection was calculated using the binding assay result wizard (Biacore control software 3.0).

Kinetic analysis was then performed using the MAGP-1 bound chips and the fibrillin-1 fragments PF1 and PF4 that were shown in analyte

Figure 1. A, schematic diagram of the domain structure of fibrillin-1, including a key showing the different domains, N-linked glycosylation sites, and C-terminal furin cleavage site. All expressed fibrillin-1 protein fragments are shown below, indicating the fibrillin-1 domains they contain. B, SDS-PAGE showing recombinant fibrillin-1 fragments, PF1, PF2, PF5, PF6, and PF7, run under reducing and nonreducing conditions and PF8 and PF13 run under reducing conditions. C, lanes 1–3 show a Western blot of PF2pro and PF2Δpro truncC. The third lane shows the unseparated second medium collection purification PF2 (2nd prep) containing PF2 and PF2Δpro truncC. Lanes 4–6 show SDS-PAGE of purified PF2Δpro truncC, PF3, and PF3Δpro. D, SDS-PAGE run under nonreducing and reducing conditions of recombinant MAGP-1 expressed using mammalian 293-EBNA cells. The recombinant protein was purified under denaturing conditions using Ni²⁺-chelate affinity chromatography and refolded by dialysis in the presence of oxidized and reduced glutathione. The MAGP-1 purified runs mainly as a monomer under nonreducing conditions, but some higher order disulfide-linked species are present.
screening to bind MAGP-1. PF9 and PF12 were also tested as negative controls. Protein fragments were injected at concentrations ranging from 0.25 to 25 μg/ml at a flow rate of 30 μl/min. Samples were injected for 3 min, dissociated for 15 min, regenerated for 1 min using HBS-Ca containing 400 mM NaCl, and then stabilized for 20 min before the next injection. After subtraction of each response value from the blank cell, association and dissociation rate constants were determined by separate $k_a$/$k_d$ fitting to the steepest parts of the binding and dissociation curves. All curves were fitted using 1:1 Langmuir association/dissociation model (BIAevaluation 3.0; Biacore AB). A saturation binding curve was also plotted by performing a four-parameter mathematical fit to calculate the top of the sensorgram curve at infinite time ($R_i$); this was possible because all of the sensorgrams leveled off before the injection end. The $R_i$ value was then plotted against concentration, and the $K_D$ and $B_{max}$ values could be calculated using nonlinear regression (one-site binding (hyperbola) GraphPad Prism version 2.0).

To investigate the effects of EDTA on binding of PF1 to MAGP-1, kinetic analysis was also performed using 40 mM PF1 and 0.01–10 mM EDTA.
EDTA in HBS-Ca. An inhibition curve was then calculated using the $R_i$ value of each curve, as described above. Similar experiments were conducted to assess the potential effects of EDTA on binding of PF5 and PF7 to tropoelastin. To determine whether direct binding of MAGP-1 to the chip influenced its ability to bind fibrillin-1, MAGP-1 was also bound to the surface of a CM5 sensor chip, at saturating levels, using an antibody to tropoelastin (R3515; Elastin Products Inc.). Fibrillin-1 fragments were passed over the antibody-bound MAGP-1 at concentrations of 200 and 1000 nM, as described for the directly immobilized MAGP-1.

Using CM5 chips with tropoelastin immobilized, kinetic analysis of tropoelastin interactions were performed using all of the fibrillin-1 fragments and MAGP-1, as analytes, at concentrations ranging from 0.5 to 60 μg/ml at a flow rate 30 μl/min. Samples were injected for 3 min and dissociated for 15 min, as described for MAGP-1, except that the regeneration step used two 30-s injections of 1 M NaCl, 50 mM NaOH, and then stabilization was for 20 min before the next injection. The blank cell response was subtracted, and association and dissociation rate constants were calculated separately, as described for MAGP-1.

Cross-linking of Tropoelastin and Fibrillin-1 Using Transglutaminase—1 μg of tropoelastin was incubated with 1 μg of fragments scanning the entire fibrillin-1 molecule, with and without 0.1 μg of transglutaminase (guinea pig liver, Sigma) for 2 h at 30 °C. This was performed in a volume of 10 μl to give protein concentration of 2 μM for both fibrillin and tropoelastin. Samples were then resolved by SDS-PAGE and transferred onto nitrocellulose. Tropoelastin was detected using a mouse monoclonal antibody BA-4 (Sigma).

Mass Spectrometry Analysis of Transglutaminase-cross-linked Tropoelastin—To analyze the cross-linked complexes of tropoelastin and fibrillin-1 by mass spectrometry, 10 μg each of fibrillin-1 fragment PF2 and tropoelastin were incubated with 1 μg of transglutaminase in a volume of 30 μl (12.5 μM for each protein) for 2 h. Ni²⁺-chelate resin was then added in order to purify the PF2 and PF2-tropoelastin heterodimers and larger cross-linked complexes from the mixture. The resin was then washed twice in 200 μl of 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CaCl₂ (TBSCaCl₂) followed by centrifugation to remove unbound noncross-linked tropoelastin and the transglutaminase; bound fibrillin-1 fragments, with or without cross-linked tropoelastin, were then eluted in 5% 2-mercaptoethanol. Samples were reduced by the addition of 9 μl dithiothreitol and incubated at 50 °C for 30 min, followed by alkylation at room temperature by the addition of 20 μl of 50% iodoacetamide for 15 min. Samples were then resolved using SDS-PAGE. Bands were visualized by colloidal Coomassie stain (Sigma), excised using a clean scalpel, and cut into 1-mm sections. The gel slices were washed twice in 200 μl of H₂O, followed by dehydration using 200 μl of 50% acetonitrile. The slices were quickly washed in 40 μM ammonium bicarbonate before the addition of 20 μl of 40 μM ammonium bicarbonate, 0.2 μg of sequencing grade trypsin (Promega). The gel slices were incubated at 37 °C overnight. For digestion using chymotrypsin after dehydration, gel slices were washed and then incubated in 100 μl Tris, pH 7.8, 10 mM CaCl₂, 0.2 μg of chymotrypsin (Roche Applied Science) and incubated at 25 °C overnight. Digestion using endoprotease Asp-N was performed in 10 mM Tris/HCl, pH 7.5, 0.2 μg of endoprotease Asp-N (Roche Applied Science). After digestion and centrifugation, the digestion buffer supernatant was removed and saved, whereas further tryptic peptides were extracted twice more in 20 μl of 70% acetonitrile for 15 min. All supernatants were then pooled, and the acetonitrile was removed by speed vacuum, until the volume was reduced to 10–20 μl. 0.1% formic acid was then added, prior to analysis using a μQ-TOF mass spectrometer (Micromass). Samples were also desalted using ZipTip C18 pipette tips (Millipore) prior to analysis by MALDI-TOF mass spectrometry.

**RESULTS**

**Solid Phase Binding of Fibrillin-1 to Tropoelastin**—In order to determine how tropoelastin interacts with fibrillin-1, solid phase binding assays were conducted at 37 °C, with tropoelastin bound to the wells and binding of soluble biotinylated or nonbiotinylated fibrillin-1 fragments detected. Fibrillin-1 fragment PF2, which contains the proline-rich region, fragment PF5, fragment PF7, and fragment PF13 showed strongly significant binding to tropoelastin (Fig. 2A). Fragments PF2 and PF5, which span fibrillin-1 exons 9–25, showed dose-dependent and saturating binding (Fig. 3, A and B). Fibrillin-1 fragments PF11 and FP12 bound more weakly to tropoelastin.

To localize further the fibrillin-1 sequence within the PF2 fragment that binds to tropoelastin, deletion constructs were prepared (Fig. 2, B and C). PF2 fragments that lacked the proline-rich region (PF2Δpro) or were truncated at the C terminus with removal of TB2 and with the proline region present or absent (PF2 trunc and PF2Δpro trunc, respectively) (see Fig. 1, A and C) were used in solid phase binding assays. Binding of PF2Δpro to tropoelastin was markedly decreased relative to the binding of PF2 to tropoelastin but was not ablated. However, neither PF2 trunc nor PF2Δpro trunc bound tropoelastin. Interestingly, PF3 (a larger fibrillin-1 fragment that encompasses PF2 and N-terminal upstream sequence encoded by exons 1–8; see Fig. 1) did not bind tropoelastin, but PF3Δpro

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**Table 1**

**Analysis of interactions between tropoelastin (TE) and fibrillin-1 protein fragments by surface plasmon resonance**

| Protein fragment | $k_a$ | $k_d$ | $K_D$ |
|------------------|-------|-------|-------|
| TE-PF1           | NB    | NB    | 10.3  |
| TE-PF2           | 1.3±0.3 | 0.28±0.05 | 279±125 |
| TE-PF5           | 27.3±6.6 | 0.11±0.02 | 4.05±0.60 |
| TE-PF6           | 8.8±2.1 | 1.12±0.2 | 151±18.3 |
| TE-PF7           | 44.4±10.3 | 0.18±0.02 | 4.95±1.49 |
| TE-PF8           | NB    | NB    | NB    |
| TE-PF9           | NB    | NB    | NB    |
| TE-PF10          | NB    | NB    | NB    |
| TE-PF12          | NB    | NB    | NB    |
| TE-PF13          | 22.6±6.5 | 0.85±0.09 | 51.9±7.4 |

**Fig. 3. Solid phase binding assays of soluble biotinylated fibrillin-1 fragments PF2 and PF5 to immobilized tropoelastin.**

Tropoelastin was coated to the plastic surface of multwell plates at 5 μg/ml and incubated with fibrillin fragments (0–50 μg/ml) at 57 °C. A, fibrillin-1 fragment PF5 (■) and nonspecific binding to BSA (●). B, fibrillin-1 fragment PF5 (■) and nonspecific binding to BSA (●). Results are shown as the mean±S.E. of triplicate values.
bound weakly to tropoelastin. These experiments show that fibrillin-1 TB2 (encoded by exons 16 and 17) is essential for tropoelastin binding to fragment PF2, that the proline-rich region influences but is not essential for binding, and that the additional presence of the N-terminal region (encoded by exons 1–8) completely inhibits tropoelastin binding.

Any possible requirement for calcium in the strong interactions detected between fibrillin-1 fragments PF2, PF5, PF7, and PF13 with tropoelastin was tested in further solid phase assays. There were no apparent differences in the binding of these fibrillin-1 fragments to tropoelastin in the presence or absence of 10 mM EDTA (Fig. 2D), indicating that tropoelastin binding to these fibrillin-1 sequences is calcium-independent.

**Biacore 3000 Analysis of Fibrillin-1 Binding to Tropoelastin**—In order to compare the solid phase binding interactions with those detected by surface plasmon resonance and to determine binding affinities, potential binding of fragments covering the entire fibrillin-1 molecule to tropoelastin was investigated using surface plasmon resonance on a Biacore 3000 instrument. In these experiments, tropoelastin was immobilized on a CM5 sensor chip (12,100 resonance units), and soluble fibrillin-1 was the analyte. Kinetic analysis was performed using all fibrillin-1 fragments at a range of concentrations (0.5–60 μg/ml) on the tropoelastin-immobilized chip. The association ($k_a$) and dissociation rate constants ($k_d$) of the binding interactions of fibrillin-1 fragments to tropoelastin and the dissociation constant ($K_D$) were determined for all fragments that exhibited any significant interaction. The kinetic data are summarized in Table I. Both PF5 and PF7 interacted very

**Table II**

| Interaction | $k_a$          | $k_d$          | $K_D$          |
|-------------|----------------|----------------|----------------|
| MAGP-1 PF1  | $110 \pm 12$   | $1.73 \pm 0.06$| $169 \pm 20$   |
| MAGP-1 PF4  | $101 \pm 27$   | $1.20 \pm 0.08$| $171 \pm 44$   |
| TE-MAGP-1   | $50.0 \pm 18.2$| $0.57 \pm 0.02$| $22.1 \pm 7.1$|
strongly with tropoelastin, with similar kinetic values (Fig. 4, A and B). The average $K_D$ of three separate binding experiments for both fragments, using the BIAevaluation software analysis, was $4.1 \pm 0.6$ and $5.0 \pm 1.5$ nM, respectively. A three-domain fragment, PF6, which includes the two overlapping domains of PF5 and PF7, also bound tropoelastin strongly, albeit with lower affinity ($K_D$ of $151 \pm 18$ nM) (Fig. 4C). PF13 (furin-cleaved) also bound tropoelastin, with lower affinity, with a $K_D$ of $51.9 \pm 17.4$ nM. Fragment PF2, which bound strongly in solid phase assays, exhibited very low binding by surface plasmon
resonance, with a $K_D$ of 279 ± 125, which may reflect differences in presentation of bound tropoelastin.

Similar Biacore binding experiments were conducted in the presence of up to 10 mM EDTA in order to determine whether the binding to PF5, PF6, and PF7 was calcium-dependent. Binding to these fragments was unaffected by EDTA (data not shown).

**Biacore 3000 Analysis of MAGP-1 Binding to Tropoelastin**—Kinetic analysis was performed using MAGP-1 as analyte at a range of concentrations (0.5–60 µg/ml) and a tropoelastin-immobilized chip (Fig. 4D). The association ($k_a$) and dissociation rate constants ($k_d$) were determined using separate fits. The average dissociation constant ($K_D$) of the interaction between MAGP-1 and tropoelastin was 22.1 ± 7.1 nM (Table II).

**Transglutaminase Cross-linking of Fibrillin-1 to Tropoelastin**—Co-incubation experiments were conducted between each of the fragments spanning the entire fibrillin-1 molecule with tropoelastin, in the presence or absence of transglutaminase, in order to determine whether any specific intermolecular transglutaminase-cross-linked complexes were formed. SDS-PAGE revealed that tropoelastin and PF2 formed unique nonreducible complexes, mainly heterodimers but also some higher order species (Fig. 5, A and B). None of these bands was present in transglutaminase-treated PF2 or tropoelastin alone. The higher order species became more prominent after longer incubations. No other fibrillin-1 fragments formed cross-linked complexes with tropoelastin.

Mass spectrometry analysis was conducted, after trypsinization, on the unique cross-linked heterodimers that had been purified by incubation with Ni$^{2+}$-chelate resin. The His$_6$ tag on PF2 bound the resin, thereby facilitating removal of transglutaminase and uncross-linked tropoelastin. Both tropoelastin and PF2 tryptic peptides were detected in the heterodimers. No tropoelastin was seen in the tropoelastin-only control samples after Ni$^{2+}$-chelate resin purification. Thus, the tropoelastin present in the transglutaminase-treated PF2 and tropoelastin preparations was cross-linked to PF2.

The tryp tic peptides detected in the heterodimers indicate fibrillin-1 and tropoelastin sequences that are not involved in cross-linking, because, if they were, they would no longer have masses recognized by the data bases. Cross-linked fragments may also be too large for effective fragmentation and MALDI-TOF or Q-TOF analysis. Two exceptions are any tryptic peptides with carboxylic charge attached to the NVT N-glycosylation consensus sequence at the end of the proline-rich region in PF2, which would not be detected due to increased mass, and the proline-rich region, which is not cleaved by trypsin. Peptides were also generated from PF2–tropoelastin heterodimers by cleavage using chymotrypsin and endoproteinase Asp-N in order to generate different fragments. A drawback with these latter enzymes is that peptides generated are less detectable by mass spectrometry, since they do not have a charged Arg or Lys at their C termini, which would facilitate flight in the mass spectrometer.

Tryptic fragments detected by mass spectrometry of the PF2 monomer band alone accounted for 49% of the sequence of this fragment, and the PF2–tropoelastin heterodimer band also gave 49% coverage of the PF2 sequence (Fig. 5C). Tropoelastin, which is lysine-rich, is likely to contribute the lysine to the cross-link because all of the lysine residues in PF2 encoded by exons 12–17 are either included within peptides recognized by mass spectrometry or trypsin cleavage immediately after them; fibrillin-1 fragments encoded by exons 9–11 can be excluded, because the fibrillin-1 fragment encoded by exons 1–11 does not form a cross-linked product with tropoelastin. No peptides encoding fibrillin-1 glutamines in exon 12 (QGSYCQ) and exon 17 (QPQPAQNSAEYQ) were detected in the heterodimers, so one or more of these glutamine residues are strong candidates for the cross-linking site (Fig. 5C). Since tropoelastin binding to PF2 is abolished when TB2 (encoded by exons 16,17) is deleted (Fig. 2, B and C), the cross-link is likely to be within TB2. Taken together, the data show that one or more glutamines encoded by exon 17 are cross-linked. Molecular modeling revealed that they are on the TB2 domain surface (Fig. 5D).

**Solid Phase Binding of Fibrillin-1 to MAGP-1**—Solid phase assays were used to identify any regions of fibrillin-1 that bind to MAGP-1. In initial experiments, wells were coated with 5 µg/ml recombinant fibrillin-1 fragments and then incubated with 5 µg/ml soluble recombinant MAGP-1. Fibrillin-1 fragment PF1 bound most strongly to MAGP-1, followed by PF5 and then PF7, but PF2 bound only weakly (Fig. 6, A and B). No other region of fibrillin-1 bound to MAGP-1. PF1 and PF5 both bind MAGP-1 in a dose-dependent and saturating manner (Fig. 6C).

**MAGP-1 Biacore Analysis**—Analyte screening of fibrillin-1 protein fragments spanning the length of the molecules for binding to MAGP-1 was performed using surface plasmon resonance (Biacore 3000). Fibrillin-1 fragments, at concentrations of 40 and 200 nM, were passed over MAGP-1, which was immobilized on the CM5 sensor chip. Both concentrations gave the same pattern of response (Fig. 7, A and B). Only the protein fragments PF-1, PF3, and PF4, all of which are at the N terminus of the molecule.
terminus of fibrillin-1, showed significant binding responses (Fig. 7C).

The association (k_a) and dissociation rate constants of these MAGP-1 and fibrillin-1 interactions and the dissociation constant (K_D) were determined by surface plasmon resonance. The kinetic data from these experiments are summarized in Table II. Both PF1 and PF4 interacted with MAGP-1 with similar kinetic values. The K_D of PF1 using the BIAevaluation software was 169 ± 20 nM, and the K_D value by saturation binding curve analysis was 242 ± 24 nM, whereas the K_D of PF4 was 171 ± 44 and 139 ± 39 nM. These data confirm that the main fibrillin-1 binding site for MAGP-1 is located within PF 4 (exons 1–8). Interestingly, no interaction was seen with PF5, although this interaction was observed in solid phase assays, possibly due to differences in presentation of bound MAGP-1 on chips or plastic wells.

To investigate whether a different MAGP-1 immobilization method would allow an interaction with PF5 using the Biacore, MAGP-1 was presented in saturating amounts on the sensor chip surface via an immobilized polyclonal antibody to MAGP-1. Fibrillin-1 fragments were then passed over the sensor chip at concentrations of 200 and 1000 nM. Using this method, some interaction was seen with PF5, but this was quantitatively less than that seen with PF1 (not shown).

To investigate the effects of EDTA on the binding of PF1 to MAGP-1, kinetic analysis was performed using 40 nM PF1 and 0.01–10 mM EDTA in HBS-Ca. An inhibition curve calculated using the R_i value of each binding curve showed strong inhibition by EDTA with an IC_50 of 1.5 mM (not shown).

**Competition Binding Assays**—Having shown that fibrillin-1 fragment PF2, but not MAGP-1, specifically binds to tropoelastin, and since it has previously been reported that MAGP-1 binds to tropoelastin (10), we used competition binding assays to investigate whether MAGP-1 and PF2 have overlapping or separate binding sites for tropoelastin (Fig. 8). In these experiments, tropoelastin was first immobilized on the wells and then preincubated with MAGP-1 or PF2, prior to incubation with biotinylated PF2 or with MAGP-1, respectively. Biotinylated PF2 bound similarly to tropoelastin and to tropoelastin that been preincubated with MAGP-1. MAGP-1 bound similar...
Tropoelastin was coated to the plastic surface of multiwell plates at 5 μg/ml, and then, after blocking with BSA, either unbiotinylated MAGP-1 or fibrillin-1 fragment PF2, respectively, was prebound at 5 μg/ml before the addition of biotinylated PF-2 or MAGP-1, respectively (shown in gray). These wells were compared with other wells where no unbiotinylated protein was added after BSA blocking but before the addition of biotinylated protein (shown in black). The direct binding of biotinylated MAGP-1 to PF2 is also shown. Results are shown as the mean ± S.E. of triplicate values.

The molecular basis of how fibrillin microfibrils act as a template for tropoelastin during elastic fibrillogenesis is a critical yet still poorly understood process in the assembly of functional elastic tissues (2). In this study, we have screened the entire fibrillin-1 molecule for sites of interaction with tropoelastin and the major microfibril-associated molecule MAGP-1. Delineation of these interactions and their kinetics has provided important new insights into this complex process. We also addressed the major issue of how tropoelastin is stabilized on microfibrils following an initial molecular association. A novel specific transglutaminase cross-link between fibrillin-1 and tropoelastin was identified that could covalently stabilize newly deposited tropoelastin on microfibrils. Identification of the high affinity binding of tropoelastin to fibrillin-1 and the cross-link thus fill a major gap in our understanding of elastic fiber formation. These data, together with our previously determined structure of fibrillin-rich microfibrils (28), provide the basis for an updated model of elastic fiber assembly (Fig. 9).

The identification of novel high affinity interactions between tropoelastin and two overlapping fibrillin-1 fragments (PF5 and FF7) encoded by central fibrillin-1 sequences (exons 18–25 and 24–30) confirms this region of fibrillin-1 as the major tropoelastin binding site. Binding of tropoelastin to PF5 and PF7 is calcium-independent, suggesting that the binding site(s) may be in TB3, which is present in both fragments. Indeed, a smaller fragment (PF6) that includes the overlapping TB3 and flanking cbEGF domains was also found to bind tropoelastin strongly in a calcium-independent manner. Its relatively weaker affinity than PF5 implies that upstream cbEGF domains stabilize the TB3 conformation and possibly also provide additional weaker interactions. SDS-PAGE analysis revealed that the PF6 band was less compact than the longer PF5 band, which contains the PF6 sequence, implying that the three-domain fragment conformation was less ordered than that of the longer PF6 (Fig. 1B). Additional evidence comes from our cell adhesion studies of the homologous RGD-containing fibrilin-1 TB4 motif. TB4 binds cells through integrins, but where TB4 is the first domain, the binding is much less than in fragments where the TB4 is preceded by six cbEGF domains (22).3 We previously showed, from three-dimensional fibrillin-1 structure analysis and antibody epitope mapping, that a fibrillin-1 region encoded by exons 16–22 locates to an exposed interbead “shoulder” feature comprising two prominent arms emerging from the bead. Our fibrillin model from these data predicts that the domains encoded by exons 18–25 and 24–30 contribute to this interbead feature (28). We previously showed that mass accretes onto the interbead “shoulder” in developing elastic tissues (29), presumably reflecting tropoelastin deposition. Mutations that cause severe neonatal Marfan syndrome (6) map within this central fibrillin-1 region, so any of these mutant molecules that are secreted and assembled could affect tropoelastin deposition.

A further interaction of moderate affinity between tropoelastin and a fibrillin-1 fragment (PF2; encoded by exons 9–17) that includes the proline-rich region has been identified by solid phase assays, although only a weak interaction was detected by Biacore. This interaction confirms an earlier report (14). Binding of tropoelastin to this fragment required the presence of the eight-cysteine motif TB2, so the binding site must be within this motif. The additional presence of N-terminal domains (encoded by exons 1–8) (PF3 fragment) ablates binding, so these domains must mask this binding site, possibly as a consequence of folding at the proline-rich region and direct interactions with TB2. Since removal of the proline-rich region (PF2Δpro) does not ablate tropoelastin binding, it cannot be the primary interaction site. However, the reduced binding to this deletion fragment suggests long range conformational changes. Since deletion of the proline-rich region from the longer fragment (PF2Δpro) does not fully rescue tropoelastin binding, there must be other upstream sequence effects. The physiological relevance of the tropoelastin interaction with C-terminal PF13 is unclear, since this fibrillin-1 sequence is almost certainly embedded within the bead (23, 28).2

3 D. Bax and C. M. Kielty, unpublished data.
We also identified a new specific transglutaminase cross-link between tropoelastin and fibrillin-1 that reinforces the critical importance of high affinity tropoelastin binding to the central region of fibrillin-1. Transglutaminases catalyze the formation of an isopeptide γ-glutamyl-ε-lysine bond either between or within polypeptide chains. Tissue transglutaminase (tTG, TG-2, TG-3) is important in stabilizing developing extracellular matrices including lung and bone and in wound repair (30, 31). The cross-link site on fibrillin-1 was localized to three glutamates within the fibrillin-1 TB2 domain (exon 17), which are on the domain surface. This cross-link may covalently link newly bound tropoelastin on microfibrils during early elastic fiber formation. Other cross-links within microfibrils and between elastin molecules within the elastin core have previously been described, but hitherto it has not been known how newly deposited tropoelastin becomes stably associated with microfibrils. Lysyl oxidases catalyze the formation of lysyl-derived desmosine and isodesmosine cross-links between elastin molecules within the core of elastic fibers (32). Microfibrils are extensively stabilized by disulfide bonds, such that reducing conditions are needed to extract fibrillin-1 molecules from fetal issues (8). A fibrillin-1 transglutaminase-cross-linked fragment of two fibrillin-1 peptides with sequences starting at residues 580 (encoded within exon 14) and 2312 (encoded within exon 56) was identified in the interbead fraction of amnion microfibrils (56) was identified in the interbead fraction of amnion microfibrils (33). MAGP-1 is also a substrate for transglutaminase activity work.

In summary, we propose an updated model of elastic fiber formation based on our new data (Fig. 9). Microfibrils are first accreted onto microfibril bead surfaces via an interaction with N-terminal fibrillin-1 domains. Tropoelastin is then deposited on an interbead region adjacent to the beads through strong interactions with the fibrillin-1 central sequence and subsequently becomes cross-linked to fibrillin-1. Juxtaposition of MAGP-1 and tropoelastin on the microfibril scaffold allows the possibility in situ of a ternary complex formation between the three molecules. This model thus provides a molecular explanation for the initial stages of elastic fiber formation.

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