Interaction of Tropoelastin with the Amino-terminal Domains of Fibrillin-1 and Fibrillin-2 Suggests a Role for the Fibrillins in Elastic Fiber Assembly*

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Alignment of tropoelastin molecules during the process of elastogenesis is thought to require fibrillin-containing microfibrils. In this study, we have demonstrated that amino-terminal domains of two microfibrillar proteins, fibrillin-1 and fibrillin-2, interact with tropoelastin in solid phase binding assays. The tropoelastin-binding site was localized to a region beginning at the glycine-rich and proline-rich regions of fibrillin-2 and fibrillin-1, respectively, and continuing through the second 8-cysteine domain. Characterization of the binding requirements using the fibrillin-2 construct found that a folded, secondary structure was necessary for binding. Furthermore, binding between tropoelastin and fibrillin was mediated by ionic interactions involving the lysine side chains of tropoelastin. The importance of the lysine side chains was corroborated by the finding that the fibrillin-2 construct did not bind to mature elastin, whose lysine side chains have been modified to form cross-links. Interestingly, there was no interaction between the fibrillin constructs and tropoelastin in solution phase, suggesting that binding of tropoelastin to a solid substrate exposes a cryptic binding site. These results suggest that fibrillin plays an important role in elastic fiber assembly by binding tropoelastin and perhaps facilitating side chain alignment for efficient cross-linking.

Elastic fibers play a crucial role in normal organ function, especially in blood vessels and the lung. Functional elastin is an insoluble matrix of polymerized monomeric subunits termed tropoelastin. Tropoelastin exhibits a modular domain structure consisting of repeating hydrophobic sequences, which contribute to the elasticity of the molecule, and lysine-rich regions that are involved in intra- and intermolecular cross-linking (1). Cross-linking of tropoelastin monomers is initiated when the ε-amino groups of lysine residues within tropoelastin become oxidized by the copper-requiring enzyme lysyl oxidase. Subsequent non-enzymatic condensation of modified and unmodified lysines then form the characteristic bifunctional and tetrafunctional cross-links specific to insoluble elastin (2, 3). Proper cross-linking is essential for elastin function as is demonstrated by weak and mechanically compromised elastic tissue associated with lathyrisms, copper deficiency, and in the disease Menkes’ syndrome (4), conditions that effect the amount or the activity of lysyl oxidase.

As assessed by immunohistochemical and immunoelectron microscopic techniques, tropoelastin always co-localizes with extracellular matrix structures termed microfibrils. Microfibrils are 10–12-nm filaments first identified as obligatory components of elastic fibers, although they are also found in non-elastic tissues, such as in the ciliary zonules of the eye (5). Rotary shadowing electron microscopy reveals microfibrils as linear arrays of electron dense “beads” separated by ~50 nm with filamentous “strings” (6–8).

Microfibrils are comprised predominantly of two proteins, fibrillin-1 (fib-1)1 and fibrillin-2 (fib-2) (9–11). Fib-1 and fib-2 have significant similarity in primary sequence and predicted protein domains (11–15). For example, each possesses tandem arrays of EGF-like and 8-cysteine (8-Cys) domains, the latter being first described in latent transforming growth factor-β-binding protein-1 (14). There is one region of each protein, however, that is distinctive; exon 10 of fib-1 encodes a proline-rich region, whereas the analogous exon in fib-2 is rich in glycine. These domains are found in similar positions within the fibrillin proteins.

Although the function of microfibrils in the extracellular space is not fully characterized, one role is to act as a molecular scaffold for the deposition and alignment of tropoelastin molecules (1, 15). This alignment presumably allows for the proper cross-linking and maturation of the elastin fiber. The prevalence of the fibrillins within microfibrils makes them candidates for the binding and alignment of tropoelastin molecules. We have assessed the ability of tropoelastin to bind to recombinantly expressed, amino-terminal fragments of fib-1 and fib-2 using solid phase binding assays and co-immunoprecipitation experiments, focusing on domains where fib-1 and fib-2 sequences are most divergent, the proline-rich region in fib-1 and the glycine-rich domain in fib-2. We were interested especially in fib-2 since it has been suggested that fib-2 preferentially localizes to elastin-rich matrices (11). We have determined that a tropoelastin-binding domain is contained within

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1 The abbreviations used are: fib-1, fibrillin-1; fib-2, fibrillin-2; 8-Cys, 8-cysteine domain; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreito1; DME,M, Dulbecco’s modified Eagle’s medium; bp, base pair; HPLC, high pressure liquid chromatography.

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the amino-terminal half of fib-2, and we have mapped at least one active site to a region that contains the Gly-rich region, downstream EGF-like loops, and most of the second 8-Cys domain. Binding to tropoelastin was not unique to fib-2, however, since the analogous region of fib-1 also interacted with tropoelastin. Interestingly, neither fibrillin fragment interacted with tropoelastin in solution phase when co-expressed in CHOK1 cells, suggesting that a cryptic binding site is exposed when tropoelastin binds a two-dimensional surface such as a plastic dish or, perhaps, a highly insoluble fibrillin-containing microfibril.

**EXPERIMENTAL PROCEDURES**

**Materials**

All materials were obtained through Sigma unless otherwise stated. Oxalic acid-solubilized bovine elastin (α-elastin) was purchased from Elastin Products Co. (Owensville, MO). Tissue culture reagents and supplements were provided by the Washington University Tissue Culture Support Center. Recombinant human tropoelastin produced in bacteria was purified as described (16).

**Fibrillin Constructs**

Fibrillin-2 Constructs—NLR3e/6 and Aik have been described previously (17). NLR3/6 consists of fib-2 sequences from bp -144 to +3542 (Met-1 to Thr-1114), whereas Aik spans bp +1274 to +2256 (Gly-425 to Phe-752) (Fig. 1). Fib-2 cDNA sequences can be accessed by GenBank® (Met-1 to Gln-46) at their amino termini, which serves as a leader construct and its expression in CHOK1 cells has been described (19). Tropoelastin Binding to Fibrillin

**Polyclonal Antibodies**

Antibody against Aik was generated using bacterially expressed Aik protein (17). The anti-Gly antibody (αGly) was raised against a fragment of the fib-2 cDNA construct, Aik, spanning bp +1274 to +1458 (Gly-425 to Asp-496), which contains most of the unique glycine-rich region specific for fib-2. A BamHI/EcoRV fragment of Aik/pBS.SK− was cloned into pGEX2T accession number L13923. Cloning of the LEEC construct and its expression in CHOK1 cells has been described (19). Both Aik and PET contain the fib-2 sequence from bp -201 to +137 (Met-1 to Gln-46) at their amino termini, which serves as a leader sequence for secretion (17). Expression of NLR3e/6, Aik, and PET in CHOK1 cells using the pEE14 mammalian expression vector (20) is described elsewhere (17).

**Western Blot Analysis**

Serum-free media from 24- to 28-h incubations with transfected cells were centrifuged to remove cell debris, and aliquots were run on polyacrylamide gels containing SDS-PAGE gels. All samples contained 50 mM dithiothreitol (DTT). Transfer of proteins to nitrocellulose and acrylamide gels containing SDS-PAGE gels. All samples contained 50 mM dithiothreitol (DTT). Transfer of proteins to nitrocellulose and incubation with antibodies were described by Trask et al. (17). Tropoelastin-specific antibodies were raised against human tropoelastin expressed in bacteria and purified over a reverse-phase C-18 HPLC column (16).

**Assessment of N- and O-Linked Glycosylation**

To determine whether the recombinant proteins contained N-linked sugars, confluent P100 dishes of transfected cells were incubated in the presence or absence of 5 µCi/ml [35S]sulfate (specific activity <100 µCi/mmol, ICN) for 5 h in sulfate- and serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate and 100 µM each of non-essential amino acids. Cells were cultured separately in the presence of 40 µCi/ml [35S]cysteine (specific activity >800 µCi/mmol, ICN, Costa Mesa, CA) in cysteine- and serum-free DMEM as a positive control. The conditioned medium was centrifuged to pellet any detached cells. To the medium was added BSA to 10 µg/ml, protein A-Trisacryl beads (Pierce), and polyclonal antibody at a final concentration of 25–50 µg/ml. Immunoprecipitation was done at 4 °C overnight. The Trisacryl beads were washed four times with PBS, 0.5% Tween 20 and then eluted in SDS-PAGE sample buffer containing DTT.

To inhibit the sulfation of Aik, potassium chloride was added to the medium prior to conditioning to a final concentration of 1.5 mg/ml per treatment. To confirm inhibition, cells were radiolabeled with [35S]sulfate for 5 h before immunoprecipitation. For non-sulfated Aik used in the binding assay, the cells were allowed to condition the sulfate-free medium overnight with 2–3 treatments of chloride without radiolabel. This medium was used for the binding assays with BSA added to 5 mg/ml. To control for possible direct effects of chloride on binding to tropoelastin (rather than via inhibition of the sulfation of Aik), chloride was added to a final concentration of 1.5 mg/ml to untreated conditioned medium and incubated at 37 °C for 30 min before use in the binding assay.

**Purification of Aik and PET**

Aik and PET were concentrated from HyQ-CCM5 (HyClone Laboratories, Inc., Logan, UT) conditioned media and were purified by reverse-phase HPLC as described (17). The C4 column was equilibrated in 80% (v/v) 10 mM ammonium acetate (pH 5.5), 30% (v/v) acetonitrile. Protein was eluted using a linear gradient of 20–70% acetonitrile at a flow rate of 150 ml/h. Fractions were dried under vacuum and resuspended in PBS.

**Assessment of the Sulfation of Constructs and Immunoprecipitation**

Aik/CHO and PET/CHO cells were cultured in the presence of 150 µCi/ml [35S]sulfate (specific activity <100 µCi/mmol, ICN) for 5 h in a sulfate- and serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate and 100 µM each of non-essential amino acids. Cells were cultured separately in the presence of 40 µCi/ml [35S]cysteine (specific activity >800 µCi/mmol, ICN, Costa Mesa, CA) in cysteine- and serum-free DMEM as a positive control. The conditioned medium was centrifuged to pellet any detached cells. To the medium was added BSA to 10 µg/ml, protein A-Trisacryl beads (Pierce), and polyclonal antibody at a final concentration of 25–50 µg/ml. Immunoprecipitation was done at 4 °C overnight. The Trisacryl beads were washed four times with PBS, 0.5% Tween 20 and then eluted in SDS-PAGE sample buffer containing DTT.

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Acetylation of Tropoelastin

Tropoelastin at 1.2 mg/ml was diluted 1:1 with a saturated sodium acetate solution and kept on ice. 1 μl of acetic anhydride was added for each milligram of tropoelastin, equally distributed over four additions in 60 min on ice. After the reaction was complete, the tropoelastin was diluted in bicarbonate buffer and plated onto microtiter wells. Mock-treated tropoelastin was prepared using the same procedure but without including acetic anhydride in the saturated sodium acetate solution.

Solution Phase Assays with Aik, PET, and Tropoelastin

Human tropoelastin, cloned into the EcoRI site in the pEE14 expression vector (18 μg), was transfected into Aik/CHO and PET/CHO cells in P100 dishes using liposome technology. 48 h after transfection, the cells were incubated with 40 μCi/ml [35S]cysteine and 50 μCi/ml [4,5-3H]leucine (specific activity 40–60 Ci/mmol, ICN) for 5 h in serum-free supplemented DMEM (lacking cysteine and leucine). The medium was collected and centrifuged to remove any detached cells. The cell layer was washed three times with PBS and then lysed in 150 mM sodium chloride, 0.5% (w/v) n-octyl β-D-glucopyranoside, 50 mM Tris, 0.1 mM phenylmethylsulfonfyl fluoride, 5 mM benzamidine, 2 mM ε-aminocaproic acid, pH 7.0 (OBG buffer) at room temperature for 10 min. The cell layer was scraped from the dish and centrifuged to pellet the insoluble material. The medium and soluble cell lysate (which includes soluble extracellular matrix components) were subject to immunoprecipitation as described above using anti-Aik, anti-Pro, or anti-TE polyclonal antibodies. Both were incubated with Trisacryl/protein A beads without primary antibody (preclear) for 30 min at 37°C. Immunoprecipitation was performed for 60–90 min at 37°C. Immunoprecipitates from Aik/CHO transfectants were washed in PBS, 0.5% (v/v) Tween 20, and the PET immunoprecipitates were washed with OBG buffer. All samples were eluted from the beads in reducing sample buffer. The samples were run on SDS-PAGE gels, fixed, enhanced, and subject to fluorography.

RESULTS

Characterization of Antibody Specificity—Polyclonal antibodies specific for fib-1 or fib-2 have been difficult to obtain because of the high degree of sequence similarity between the two proteins. We addressed this problem by generating antibodies to bacterial fusion proteins containing the proline-rich domain of fib-1 and glycine-rich domain of fib-2 (see "Experimental Procedures" and Fig. 1). The Western blot in Fig. 2A shows that these polyclonal antibodies were specific for their respective antigens; the anti-antibody to the glycine-rich domain (αGly) reacted with Aik and NLR3e/6 but not PET, and similarly, the anti-Pro (αPro) antibody recognized PET but not the fib-2 constructs. Also, neither antibody reacted with proteins in the medium of untransfected CHOK1 cells.

Expression and Characterization of Fibrillin Constructs in CHOK1 Cells—Constructs containing amino-terminal regions of fib-1 and fib-2, depicted schematically in Fig. 1, were expressed in CHOK1 cells. The fib-2 construct, Aik, begins five residues into the glycine-rich region (11) and extends into the second 8-cysteine domain, ending three residues short of the 8th cysteine. PET includes the first residue of the proline-rich domain of fib-1 (12) and ends with the last residue predicted for the second 8-Cys domain. Both Aik and PET contain the putative leader sequence of fib-2 found within amino acids Met-1 to Gln-46 (see Fig. 1). NLR3e/6 contains contiguous sequences from the initiator methionine of fib-2 to the end of the first EGF-like loop of the middle stretch of 12 EGF-like domains in the full-length molecule. LEEC contains the leader sequence of fib-1 spliced onto the large central region of EGF-like domains (exons 23–44) and the carboxyl-terminal domain (exons 64–65) of fib-1. The Western blots shown in Fig. 2A demonstrate that these proteins were stably expressed in CHOK1 cells. Treatment of the cells with tunicamycin (Fig. 3A) confirmed that all constructs were N-linked glycosylated as predicted by their primary sequences. PET was O-linked glycosylated as assessed by its ability to be recognized by Jacalin, a lectin that specifically recognizes O-linked sugars (Fig. 3B). Aik and NLR3e/6, however, did not bind to Jacalin and, thus, did not contain O-linked sugars.

NLR3e/6, Aik, and PET, Specifically Bind to Tropoelastin in a Solid Phase Binding Assay—Fig. 4A shows that unpurified NLR3e/6 bound in a dose-dependent manner to tropoelastin that had been coated onto microtiter wells. There was minimal interaction of NLR3e/6 with wells blocked with non-fat milk. Aik, a smaller construct containing the front portion of NLR3e/6, also bound to tropoelastin (Fig. 4B) thus localizing at least one active region within NLR3e/6 responsible for its binding activity. No interaction was seen between Aik and bovine serum albumin or wells coated with non-fat milk indicating specificity toward tropoelastin. HPLC-purified Aik exhibited similar binding characteristics as compared with unpurified Aik/CHO conditioned medium (Fig. 4C), indicating that a direct interaction between Aik and tropoelastin was occurring. No binding was observed between tropoelastin and purified Aik expressed in bacteria (Fig. 4D), suggesting that the folded, secondary structure of Aik is critical for its binding activity.

The possible binding of tropoelastin to fibrillin-1 was investigated using the PET construct, which contains regions of fib-1 that are analogous to Aik. Fig. 4E indicates that purified PET, like Aik, bound to tropoelastin in a dose-dependent manner. To determine whether tropoelastin binding was specific for the amino-terminal region of fibrillin and not a general property of any fragment of the molecule, LEEC, a construct containing the central region and carboxyl-terminal domain of fib-1, was used as a competitor of Aik in the tropoelastin binding assays.

FIG. 1. Schematic depiction of the fibrillin-1 and fibrillin-2 constructs used in this study and their relative positions within the full-length molecules.

FIG. 2. Expression and characterization of constructs in CHOK1 cells. Western blotting on serum-free conditioned media was performed for each of the recombinantly expressed proteins using the indicated polyclonal antibodies. There was no cross-reactivity of the antibodies with either proteins from untransfected CHOK1 cells or the other fibrillin type. C, untransfected CHOK1 cells; A, Aik-producing CHO cells; P, PET-producing CHO cells; N, NLR3e/6-producing CHO cells. Antibody αPro was used to detect PET, and antibody αGly was used to detect Aik and NLR3e/6.
Because our antibodies could not recognize native LEEC, it was not possible to access direct binding of LEEC to tropoelastin using the microwell assay. As shown in Fig. 4B, concentrations of LEEC greater than 5 μg/ml did not inhibit binding of Aik to tropoelastin-coated wells, suggesting that LEEC itself does not bind tropoelastin. It should be noted that because of the propensity of tropoelastin to bind to plastic surfaces even when blocked with high levels of protein, it is not possible to do solid phase binding assays with tropoelastin in the solution phase (21) nor is it possible to determine accurately binding affinities.

**Binding of Aik and PET to Tropoelastin Is Sensitive to Ionic Conditions**—The highly charged nature of tropoelastin (pI ~13) suggests that ionic interactions may play a role in binding to fibrillin. This was investigated by conducting the binding assays in buffers of increasing ionic strength. For these assays, we used conditioned medium from the Aik/CHO cells as a source of protein. Fig. 5A shows that binding was sensitive to increasing amounts of sodium chloride, exhibiting an ~75% inhibition of binding at 400 mM. This inhibition by sodium chloride implicates a charge-charge interaction.

To assess whether the lysine side chains of tropoelastin were involved in binding, purified tropoelastin was acetylated with acetic anhydride, a chemical modification that renders amino groups uncharged. Fig. 5B shows that Aik bound ~60% less well to acetylated tropoelastin as compared with mock-treated tropoelastin. This modification did not alter the ability of tropoelastin to bind to the microtiter wells when detected with tropoelastin antibody (data not shown). Estimations of the efficiency of acetylation using this procedure indicated that only ~30% of the lysine residues of tropoelastin were modified.

**Binding of the fib-1 construct, PET, to tropoelastin was also sensitive to ionic conditions (Fig. 5A), exhibiting a ~65% inhibition at 400 mM sodium chloride. By analogy to the data obtained with Aik, we believe that PET also interacted with the unmodified lysine residues in tropoelastin.**

**Effects of Fibrillin Sulfation on Binding to Tropoelastin**—Characterization of post-translational modifications of our fib-2 constructs demonstrated that NLR3e/6 (data not shown) and Aik incorporated [35S]sulfate (Fig. 6A). Because the fib-2 constructs were not O-glycosylated (Fig. 3B), their sulfate groups were most likely on tyrosine residues or, possibly, N-linked sugars (22–24). Autoradiography of medium from PET/CHO cells incubated with [35S]sulfate showed weak labeling of PET, with much longer exposure times required to detect only minimal signal. We are uncertain whether this low level of labeling reflects fewer sulfated amino acids in PET as compared with Aik or if trace amounts of sulfate are incorporated into the sulfur-containing amino acids methionine and cysteine through cellular recycling of the [35S]sulfate. Interestingly, Kiely et al. (25) demonstrated that full-length fib-1 will efficiently incorporate [35S]sulfate. Our results suggest that the site of sulfation in fib-1 must be outside the region encoded by PET.

Because sulfation imparts a negative charge, we sought to determine if this post-translational modification was important for the interaction of Aik with the positively charged lysine residues in tropoelastin. Sulfation of proteins can be inhibited with potassium chlorate, which inhibits the synthesis of the cellular sulfate donor group, 3'-phosphoadenosine 5'-phosphosulfate (26). Treatment of Aik/CHO cells with chlorate fully prevented the sulfation of Aik (Fig. 6B) without affecting the level of protein expression as demonstrated by the Western blot in Fig. 6, C and D, which shows that non-sulfated Aik bound ~30% less well to tropoelastin implicating this modification as contributing to binding. Chlorate itself had no adverse effect on the binding assays, since adding the chemical to untreated conditioned medium did not appreciably alter the binding of sulfated Aik to tropoelastin (Fig. 6D).

**Aik Does Not Interact with Mature Elastin**—The majority of lysine residues in tropoelastin are modified to form covalent cross-links in insoluble elastin (1). Cross-link formation involves oxidative deamination of the lysyl ε-amino group resulting in a dramatic change in charge on the mature protein, the pI of mature elastin is ~6 compared with a pI of ~13 for tropoelastin. Therefore, one would predict, given the data in Fig. 5B, that Aik would not bind to insoluble, cross-linked elastin. Because mature elastin is highly cross-linked and insoluble, it cannot be used in traditional binding assays. To circumvent this problem, we assessed binding of Aik to ε-elastin. ε-Elastin is generated by treating insoluble elastin with oxalic acid (27), which randomly hydrolyzes sites in the elastin peptide backbone generating soluble, cross-link-containing tropoelastin.
Fig. 4. NLR3e/6, Aik, and PET bind to tropoelastin in a solid phase binding assay. A, NLR3e/6 from conditioned medium bound to tropoelastin coated onto microtiter wells in a dose-dependent manner (squares) but not to milk-blocked wells (circles). B, Aik from conditioned medium bound to tropoelastin (squares) but not to bovine serum albumin (triangles) or wells blocked with milk protein (circles). Open circle shows Aik binding to tropoelastin following preincubation of tropoelastin with ~50 µg/ml LEEC. C, reverse-phase HPLC-purified Aik also interacted with tropoelastin (squares), with minimal binding to blocked wells (triangles). Specific binding (circles) was calculated by subtracting the background binding to blocked wells from the average values of binding to tropoelastin at a particular concentration of Aik. D, in contrast to Aik expressed in mammalian cells, purified Aik expressed in bacteria did not bind to tropoelastin (circles). Squares show bacterial Aik binding to BSA-coated wells. E, reverse-phase HPLC-purified PET (fib-1 construct) binding to tropoelastin is shown by squares. Nonspecific binding of PET to milk-blocked wells is shown by filled circles. Specific binding of PET (triangles) was calculated as in C. Antibody αGly was used for detection in A and C, and anti-Aik was used in B and D, and antibody αPro was used in E. Bars around the symbols indicate S.D. and may not be visible if the S.D. is smaller than the size of the symbol.

Fig. 5. Characterization of the ionic dependence for interaction between the fibrillin constructs and tropoelastin. A, increasing the ionic strength by raising the sodium chloride concentration resulted in a dramatic decrease in the binding of both Aik (squares) and HPLC-purified PET (circles) to tropoelastin. The amount of Aik (final concentration, 1×) or PET (25 µg/ml) was kept constant for each data point. B, neutralizing the positive charge on lysines within tropoelastin by modification with acetic anhydride decreased the amount of bound Aik. Mock treatment refers to tropoelastin that had been subject to the modification protocol in the absence of acetic anhydride. Antibody αGly was used for detection of bound Aik.

peptides. Fig. 7 demonstrates that Aik did not bind to α-elastin in the solid phase assay.

Aik and PET Do Not Interact with Tropoelastin in Solution Phase—To characterize further the interaction between the fibrillin constructs and tropoelastin, human tropoelastin, cloned into the pEE14 expression vector, was transiently transfected into CHOK1 cells stably expressing either Aik or PET. Two days after transfection, the cells were labeled with [35S]cysteine (to label Aik or PET) and [3H]leucine (to label tropoelastin). Immunoprecipitation of the transiently expressed tropoelastin was performed at 37 °C to determine whether Aik (or PET) would bind tropoelastin in the solution phase and precipitate as a tropoelastin-Aik (or tropoelastin-PET) complex. Fig. 8, A and B, shows that neither Aik nor PET co-precipitated with tropoelastin in the transiently transfected cells. These data suggest that a cryptic site(s) is exposed when tropoelastin binds to a two-dimensional surface. This change in conformation would be required for interaction between the fibrillin constructs and tropoelastin.

DISCUSSION

The assembly of an elastic fiber is a highly regulated and complex process requiring several coordinated steps. Monomers of tropoelastin must be transported through the cell and secreted at sites for assembly near the cell surface. In the extracellular space, tropoelastin monomers must be aligned so that cross-links can form between appropriate sites (28). In considering how cross-linking occurs, a mechanism must exist to bring up to four positively charged lysine side chains into close proximity for subsequent processing (29). Microfibrils are thought to contribute to this process by aligning the tropoelastin molecules and allowing for efficient and functional cross-linking (1, 15). The results described above document a specific interaction between tropoelastin and fibrillin, supporting a role for fibrillin in elastin assembly.

By using recombinantly expressed fragments of fibrillin, we have shown that an amino-terminal region of fib-2, corresponding to ~40% of the mature protein, interacted with recombinantly expressed and purified tropoelastin in solid phase as-
Aik is post-translationally modified with sulfate moieties that are important for its interaction with tropoelastin. A, immunoprecipitation for Aik or PET from conditioned medium after labeling transfected cells with [35S]sulfate demonstrated strong labeling of Aik. The [35S]cysteine label indicated similar amounts of Aik and PET were loaded on the gel. The arrow indicates the gel position of Aik and Pet. B, an autoradiograph showing that the sulfation of Aik can be fully inhibited by treatment of the Aik/CHO cells with potassium chlorate. Shown is the immunoprecipitation of Aik from [35S]sulfate-labeled cells incubated in the presence (+) or absence (−) of chlorate. C, Western blot analysis demonstrates that the levels of Aik expression were identical between chlorate-treated and untreated Aik/CHO cells. D, conditioned medium containing Aik from chlorate-treated cells (ClO3) showed a decrease in binding of non-sulfated Aik to tropoelastin. Mock treatment refers to the addition of chlorate to untreated conditioned medium to rule out direct effects of the chlorate itself on binding rather than via its inhibition of the sulfation of Aik. Anti-Aik was used for detection in A–C, whereas antibody αGly was used in D.

Aik does not bind to cross-linked elastin. Aik did not interact with α-elastin that was coated onto microtiter wells (circles). Binding to tropoelastin (squares) is shown for reference. Anti-Aik was used for detection of bound Aik.

The fibrillin constructs do not interact with tropoelastin in solution phase. Full-length human tropoelastin (TE) was transiently transfected into Aik/CHO (A) or PET/CHO (B) cells. 48 h after transfection, the cells were radiolabeled with [35S]cysteine and [3H]leucine, and then the cell lysate (containing intracellular material and soluble extracellular matrix components) was subjected to immunoprecipitation for tropoelastin using polyclonal antibody anti-TE. Aik (A) and PET (B) did not co-immunoprecipitate with tropoelastin (TE). Immunoprecipitations for Aik (with anti-Aik) and PET (with antibody αPro) served as a positive control for their expression. Preclear refers to material precipitated by the Trisacryl/protein A beads in the absence of antibodies.

The regions of fibrillin represented by PET and Aik may be an exposed region of the protein that can interact with multiple matrix molecules. Recent studies in our laboratory have shown that PET, but not Aik, interacts with decorin, a small molecular weight proteoglycan (30). Whether decorin and tropoelastin bind to the same site in PET is not yet known.

Binding of fibrillin to tropoelastin is sensitive to ionic conditions, with maximal inhibition of binding occurring at 400 mM sodium chloride. The importance of lysine side chains to the ability of tropoelastin to interact with Aik is emphasized by the fact that acetylation of some of these residues caused nearly a 60% decrease in binding. Given our understanding of elastin cross-linking requirements, we speculate that by interacting with lysine side chains, the fibrillins may be acting to shield the charged ε-amino groups in tropoelastin so that appropriate side chains can be juxtaposed prior to oxidation by lysyl oxidase. This shielding effect could be mediated by negatively charged amino acids within the tropoelastin-binding regions of the fibrillins or by sulfate groups on amino acids or sugars. Sulfation could enhance the interaction between fibrillin and tropoelastin through the addition of negatively charged moieties in the region of fibrillin that binds tropoelastin.

The fact that the fib-2 construct, Aik, did not interact with mature elastin (α-elastin) under conditions where binding to tropoelastin was observed may provide an explanation for how tropoelastin is released from microfibrils during periods of active elastin synthesis and fiber growth. As a consequence of the cross-linking reaction, the interaction between tropoelastin and microfibrils will no longer be favorable as a result of
deamination of lysine side chains. The microfibril will then release the cross-linked elastin and bind another tropoelastin molecule, acting as a catalyst for further cross-linking. This may explain why microfibrils are found in greatest abundance on the periphery of amorphous elastin (5), where new elastin is added to the surface of the growing fiber.

It is of interest that the interaction between the fibrillin constructs and tropoelastin did not occur in solution phase when both proteins were secreted from CHOK1 cells. We interpret this finding to indicate that cryptic fibrillin interaction sites in tropoelastin are exposed as a consequence of a conformational change when tropoelastin interacts with a solid surface. By analogy, similar changes in structure may occur as tropoelastin associates with insoluble, fibrillin-containing microfibrils in the extracellular matrix. Exposing a cryptic site as a consequence of binding to insoluble matrix would limit the interaction of tropoelastin to fibrillins in assembled microfibrils. This "controlled interaction" would restrict elastic fiber assembly to places within the appropriate environment. The use of cryptic binding sites to regulate protein-protein interactions has been documented for other matrix proteins (e.g. fibronectin) (31).

Our finding that the fibrillin constructs do not associate with tropoelastin when co-expressed in CHOK1 cells is similar to earlier studies with FCL cells (32). FCL cells in culture produce abundant tropoelastin and fibrillin as well as other known microfibrillar components but do not assemble elastic fibers. Immunoprecipitation studies found that tropoelastin in the medium did not co-precipitate with fibrillin, although both proteins were secreted at high levels (32). When the FCL cells were grown on a slice of elastic ligament, tropoelastin readily associated with the matrix and was incorporated into the existing fibers (33). These findings suggest that the presence of an insoluble matrix "surface" containing assembled elastic fiber proteins was sufficient to reinitiate the assembly process, perhaps through the mechanism described above.

Binding to tropoelastin to Aik and Pet suggests that both fibrillins may play a role in the organization of the elastic matrix, a finding not surprising given the fact that the fibrillins are believed to be major components of microfibrillar architecture. Furthermore, fib-1 and fib-2 expression in development do not always overlap spatially or temporally (18), so the ability of tropoelastin to bind to both proteins may be an adaptive advantage to ensure that elastin can assemble on any microfibrillar bed. This redundancy in binding may explain why elastic fibers have been observed in vivo where homologous recombination has been used to produce a severe reduction in the amount of expressed fib-1 (34).

Elastin is a recent gene found only in vertebrates. Its appearance in evolution is sudden and coincident with the development of the closed, pulsatile, high pressure circulatory system (35). Fibrillin-containing microfibrils, in contrast, have been identified in early invertebrates (36), implying that fibrillin is an ancient protein. Because fibrillin appears in evolution much earlier than elastin, it is hard to argue that its sole function is to assemble elastic fibers. It is more likely that elastin assembly and fibrillin assembly have evolved independently. Our findings would suggest that microfibrils provide a "matrix target" for elastin assembly and initiate and perhaps control the assembly process. In the presence of microfibrils, elastin assembly might occur more efficiently and with spatial constraints imposed by the physical orientation of the microfibrillar bundle (e.g. forming sheets, fibers, or filaments). Being able to utilize either fib-1 or fib-2-containing microfibrils as a target and scaffold for tropoelastin assembly would ensure that a functional elastic fiber would be correctly positioned in the tissue matrix wherever there are microfibrils.

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