Domains in Tropoelastin That Mediate Elastin Deposition in Vitro and in Vivo*

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Elastic fiber assembly is a complicated process involving multiple different proteins and enzyme activities. However, the specific protein-protein interactions that facilitate elastin polymerization have not been defined. To identify domains in the tropoelastin molecule important for the assembly process, we utilized an in vitro assembly model to map sequences within tropoelastin that facilitate its association with fibrillin-containing microfibrils in the extracellular matrix. Our results show that an essential assembly domain is located in the C-terminal region of the molecule, encoded by exons 29–36. Fine mapping studies using an exon deletion strategy and synthetic peptides identified the hydrophobic sequence in exon 30 as a major functional element in this region and suggested that the assembly process is driven by the propensity of this sequence to form β-sheet structure. Tropoelastin molecules lacking the C-terminal assembly domain expressed as transgenes in mice did not assemble nor did they interfere with assembly of full-length normal mouse elastin. In addition to providing important information about elastin assembly in general, the results of this study suggest how removal or alteration of the C terminus through stop or frameshift mutations might contribute to the elastin-related diseases supravalvular aortic stenosis and cutis laxa.

The inherent complexity of the elastic fiber, combined with the unique physical properties of its component proteins, has made understanding elastin structure and assembly one of the most difficult problems in matrix biology. Although technical advances in cell and molecular biology have given us new information about elastin gene expression and elastin synthesis, surprisingly little is known about how the elastic fiber is assembled at the molecular level. The major component of the mature fiber is a covalently cross-linked polymer of the protein elastin. Elastin is secreted from the cell as a soluble monomer called tropoelastin. In the extracellular space, lysine residues within tropoelastin are specifically modified to form covalent cross-links between tropoelastin chains. This cross-linked polymer has a high degree of reversible distensibility, including the ability to deform to large extensions with small forces.

The tropoelastin molecule is characterized by a series of tandem repeats, each including a lysine-containing cross-linking region followed by a hydrophobic motif (1). Cross-linking is initiated by the extracellular enzyme(s) lysyl oxidase, which catalyzes the oxidative deamination of lysyl ε-amino groups. Under normal conditions, the cross-linking process is extremely efficient, with all but ~5 of the ~40 lysine residues in tropoelastin participating in covalent linkages that form the functional polymer. How the cross-linking sites within the monomer are aligned prior to cross-linking is unclear. It has long been assumed that microfibrils provide a scaffold or template for elastin assembly by binding and aligning tropoelastin monomers so that lysine-containing regions are in register for cross-linking. This idea evolved from electron microscopic images showing that the appearance of microfibrils is the first ultrastructural indication of the elastic fiber (2–4) and that microfibrils are associated with elastin throughout the elastogenic period.

Recently, two inherited diseases, autosomal dominant cutis laxa and supravalvular aortic stenosis (SVAS),1 have been linked to mutations within the elastin gene that may alter the ability of the elastin precursor to undergo normal assembly (5, 6). Each of these diseases has a distinct clinical phenotype and may be caused by fundamentally different molecular mechanisms. Autosomal dominant cutis laxa is characterized by redundant, loose, sagging, and inelastic skin with variable systemic organ involvement. The known elastin mutations in autosomal dominant cutis laxa are single nucleotide deletions in exons 30 and 32 that, depending on exon splicing, give rise to a missense peptide sequence extending into the 3′-untranslated region (6, 7). Although the exact pathomechanism of this disease is not clear, it is thought that alterations at the C terminus of the secreted mutant protein interfere with the deposition of normal elastin in a dominant-negative fashion (5, 6).

SVAS is characterized by thickening of the arterial wall and either focal or diffuse narrowing of the aorta and, frequently, of other major arteries. Mutational analysis has identified a wide spectrum of mutations associated with SVAS, including large deletions, translocations, inversions, and, most frequently, point mutations (8–12). Accumulating evidence suggests that the pathomechanism of SVAS is haploinsufficiency (13), which can result from the deletion of one complete copy of the elastin gene (as occurs in Williams syndrome) or through functional hemizygosity arising from loss-of-function mutations in one elastin allele.

1 The abbreviations used are: SVAS, supravalvular aortic stenosis; PE, pigmented epithelial; RT, reverse transcription; bTE, bovine tropoelastin; PBS, phosphate-buffered saline.

Received for publication, December 13, 2002, and in revised form, March 3, 2003
Published, JBC Papers in Press, March 6, 2003, DOI 10.1074/jbc.M212715200
PCR amplification of total RNA isolated from fetal bovine ligament cells. Fetal bovine ligament cells are known to express an alternatively spliced form of tropoelastin in which exon 33 has been spliced out (1). The PCR primers used for the amplifications include the exon 27F primer as well as the exon 36R primer that leads to amplification of the entire 36th exon as well as its stop codon (5′–GGATCTAGATCTGAGTGGCTGCTCTTCCGCGAC-3′). The product of the amplification was then cloned with NotI and XbaI and ligated into the FL+ vector cut with the same enzymes. To generate the Cys-to-Ala mutations, oligonucleotide-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) with oligonucleotides C755A-F (5′–GGAGAAATCCGTGGCGGAAAGAGC-3′) and C755A-R (5′–CTTCTCCGGCCAGGGATTTCCCA-3′) and oligonucleotides C751A-F (5′–CAATCCGTGGCGGATTTCCCA-3′) and C751A-R (5′–AATCATCTTGGCCGCGAC-3′) according to the manufacturer’s recommendations.

Several steps were required to generate construct Δ30. First, exons 28–36 were amplified by PCR. The primers used were exon 28F (5′–GGAATTCAGATCTGAGTGGCTGCTCTTCCGCGAC-3′) and the exon 29R primer (5′–GGATCTAGATCTGAGTGGCTGCTCTTCCGCGAC-3′) and the exon 36R primer listed above. The exon 28F primer contains an EcoRI site such that the product can be digested with EcoRI and XbaI and ligated into a similarly digested pUC-19 shuttle vector (pUC-28–36). To generate the Δ30 insert, exon 30 was deleted by PCR amplification of a 113-bp fragment using the exon 28F primer and the exon 29/31R primer (5′–AATCATCTTGGCCGCGAC-3′) encoding a PstI site in exon 31. The EcoRI-PstI-restricted fragment was ligated into the pUC-28–36 plasmid, resulting in the pUC30 plasmid. pUC30 was then digested with NotI and XbaI, and the TE30 insert was ligated into the NotI/XbaI-restricted FL+ plasmid, resulting in pCNeo-TE30. All alterations were verified by nucleotide sequencing.

Tissue Culture—PE cells (20) were maintained in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with nonessential amino acids (Cellgro), sodium pyruvate (Cellgro), penicillin/streptomycin (Washington University Tissue Culture Supply Center), and 10% fetal calf serum (Hyclone Laboratories). Cells were maintained in a 37 °C humidifying CO2 incubator.

Transfection—Stable transfection of PE cells was performed using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Briefly, 2 × 105 cells were plated in a six-well plate. When cells reached 60–80% confluence, 1 μg of the chosen construct and 1 μl of reagent complex were added to the cells in Opti-MEM serum-free medium (Invitrogen). Sixteen hours later, the transfection reagents were removed, and fresh Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum was added. After 48 h, cells were then placed under selection with 500 μg/ml active Geneticin (G418 sulfate, Invitrogen).

Immunofluorescence—To detect bovine tropoelastin in the matrix of stable pools of G418-selected PE cells, the cells were plated on glass coverslips in six-well plates and allowed to reach confluence. Seven days after visual confluence, the medium was removed, and the cells laid down were washed with phosphate-buffered saline (PBS) to remove all non-cell layer-associated proteins. Cells were then fixed with ice-cold methanol for 60 s and washed with PBS to remove any remaining alcohol. The cells were treated with blocking solution consisting of PBS containing 1% gelatin and 0.1% Tween 20. The anti-human recombinant tropoelastin antibody was used at a dilution of 1:250 in blocking solution. Fluorescent secondary antibody (goat anti-rabbit 488, Molecular Probes, Inc.) was used at a concentration of 1:2000 in blocking solution. After secondary antibody incubation, the cells were washed before removal from the six-well plate and inversion of the coverslip onto a glass slide. Fluorescence was protected using anti-fade mounting medium (Gelmount, Biomedia Corp.). A Zeiss Axioscope microscope was used for both fluorescence microscopy and immunoelectron microscopy. Images were captured with an AxioCam digital camera using Axiovision software. All images are shown at magnification ×40 except where noted.

**Metabolic Labeling and Immunoprecipitation**—Conditioned media from confluent monolayers of fetal bovine chondrocytes, untransfected PE cells, and stably transfected PE cells that were metabolically labeled with L-[4,5-3H]leucine (1 mCi/ml; ICN Pharmaceuticals, Irvine, CA) and cultured for 16 h were immunoprecipitated for tropoelastin as described previously (19). Immune complexes were pelleted, washed, and resuspended in 35 μl of Laemmli sample buffer containing 100 mM dithiothreitol. Samples were electrophoresed on SDS-polyacrylamide gels, fixed, treated with EN3HANCE (PerkinElmer Life Sciences) for 1 h, dried, and exposed to X-Omat AR film (Eastman Kodak Co.).
components in addition to the appropriate tropoelastin sequence. To generate construct Δ30, the pCneo construct was linearized, and extraneous plasmid sequences (neo cassette, etc.) were removed by restriction digestion with ClaI. In all cases, the appropriate fragment was purified using a QIAGEN gel extraction kit. Each linearized product contained, at a minimum, the tropoelastin sequence flanked by the cytomegalovirus immediate-early promoter and the SV40-derived 3′-untranslated region and poly(A) signal. Each fragment was resuspended at 2 μg/ml in injection buffer composed of 5 mM Tris-HCl (pH 7.4), 0.25 mM EDTA (pH 8.0), and 5 mM NaCl and was injected into B6C3F1 mouse fertilized eggs, which were implanted into the uteruses of pseudopregnant foster mothers. After birth, potential founders were screened for the presence of the transgene using PCR and bovine tropoelastin-specific forward (5′-GGGTACCAAGGACTGTCCCG-3′) and reverse (5′-CCATGCGGTTGACTCGCTGC-3′) primers. Once detected, animals positive for the transgene were mated to wild-type animals to stabilize the line.

RT-PCR—RNA was isolated from wild-type and transgenic animals using RNAzol (Tissue Tek) according to the manufacturer’s instructions. Heart, lung, kidney, and liver total RNAs were screened in all founders tested. RT-PCR was performed on 1 μg of each resultant RNA under the following conditions. Residual DNA was removed by treating the RNA with RQI RNase-free DNase in first-strand IRT reaction buffer (Invitrogen) containing 2 μl of 0.1 μM dithiothreitol for 30 min at 37 °C. The enzyme was heat-inactivated at 72 °C for 10 min. Reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions in the presence of dNTPs random primers (Roche Applied Science) and dNTPs (Promega). PCR was then performed on the resultant cDNA with bovine tropoelastin-specific forward (5′-GGGTACCAAGGACTGTCCCG-3′) and reverse (5′-CCATGCGGTTGACTCGCTGC-3′) primers. Thirty-five cycles of amplification were performed with a 67 °C annealing temperature and a 30-s extension time. The products of this reaction were then analyzed by gel electrophoresis.

Immunofluorescence Analysis—Tissues from transgenic and wild-type animals were dissected and washed with sterile PBS before being embedded in blocks containing OCT freezing medium (Tissue Tek). The tissues were then frozen over dry ice, and 10-μm sections were cut using a microtome.

For immunofluorescence analysis, sections were thawed to room temperature, fixed in cold ethanol, and etched to enhance antigenicity with 1 mg/ml hyaluronidase in 0.1 M sodium acetate buffer (pH 5.5) containing 0.85% NaCl for 30 min at 37 °C. After hyaluronidase treatment, the tissues were treated as described above for tissue culture cells. The antibodies used were BA4 and N6 (Covance) diluted 1:500 dilution. Goat anti-mouse 594 and goat anti-rabbit 488 secondary antibodies (Jackson ImmunoResearch) were used at a 1:1000 dilution. Goat anti-mouse 594 and goat anti-rabbit 488 secondary antibodies (Molecular Probes, Inc.) were used for primary antibody detection.

Quick-freeze Deep-etch Electron Microscopy and Congo Red Staining of Exon 30 Peptides—A peptide encoding exon 30 of bovine tropoelastin (GLGGVGGGGLGAVGLAGVGVGLG) was synthesized by solid-phase peptide synthesis using a 431 A peptide synthesizer (ABI) running Fast-Moc chemistry and dissolved at 10 mg/ml in 7 mM guanidine HCl. A second, scrambled version (LVGGGGGGLPVLGGAGALGGVGV) was also synthesized as a control for this assay. The peptide stock solution (10 μl) was diluted with 200 μl of PBS and 200 μl of water and subjected to slow rotation overnight at room temperature. Precipitated peptide was pelleted by centrifugation in a microcentrifuge, smeared on a glass slide, fixed in 2% (w/v) glutaraldehyde and stained with 0.05% Congo red in 50% glycerol. Evaluation of Congo red staining by polarization microscopy was performed using a Zeiss Axioscope equipped with optimally aligned cross-polarizers. For electron microscopy, freshly prepared mica flakes were added to solutions containing peptide films, followed by freeze-drying and platinum replication according to established procedures (18, 19).

Purification of Recombinant Tropoelastin—Full-length bovine tropoelastin was cloned into the pQE vector (QIAGEN Inc.), expressed in bacteria, and purified with nickel-nitrotriacetic acid resin (QIAGEN Inc.) according to the manufacturer’s instructions. Bound protein was then eluted with 6 M urea buffer (pH 4.0) and dialyzed in 0.1% acetic acid. Protein concentration was quantified by amino acid analysis, and aliquots of 200 μg were lyophilized.

Protease Treatment and Western Blotting—Aliquots (200 μg) of recombinant tropoelastin were resuspended in 350 μl of PBS containing the protease inhibitors EDTA (1 mM) and pepstatin (1 mM) to block proteolysis by non-trypsin-type proteases. A sample was taken prior to the addition of plasmin at time 0. Then, 1 μl of 0.2 μg/ml plasmin was added, and 50-μl samples were taken at 30, 60, and 120 min. Plasmin activity was stopped by adding 1× SDS-PAGE sample buffer containing 0.1 μl diithiothreitol and boiling for 5 min. The sample was electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose (Schleicher & Schuell). Nitrocellulose blots were blocked in 5% (w/v) nonfat milk in 50 mM Tris (pH 7.5), 171 mM NaCl, and 0.05% (v/v) Tween 20 (TTBS). The CT, primary antibody (18) was diluted 1:250 in TTBS containing 2.5% (w/v) nonfat milk and incubated for 1 h at room temperature. The blot was then washed and incubated with peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Amersham Biosciences) diluted 1:2000. Chemiluminescence detection was performed using ECL Western blot detection reagents (Amersham Biosciences) and exposed to XAR-5 x-ray film (Eastman Kodak Co.).

RESULTS

The Presence of the Tropoelastin C Terminus Is Required for Its Deposition into the Extracellular Matrix of PE Cells—To determine which regions of tropoelastin are necessary for its association with the extracellular matrix, we generated expression constructs consisting of full-length elastin as well as mutant and deletion forms of the molecule. These constructs were then transfected into PE cells, and the ability of the expressed transgene to associate with microfibrils in the PE cell matrix was determined by immunofluorescence microscopy. PE cells are a cell line derived from the pigmented epithelial cells in the ciliary body of the bovine eye (20). They are known to lay down an elaborate fibrillar matrix composed of fibrillin-1, fibrillin-2, and MAGP1 (microfibril-associated glycoprotein-1), but do not produce endogenous elastin (17). Because these cells produce all of the components necessary to form the scaffolding for elastic matrices, but not tropoelastin itself, they provide a useful system for studying the early stages of elastic fiber assembly.

After stable transfection of PE cells with the full-length bovine tropoelastin construct, microfibrils in the PE cell matrix became decorated with elastin expressed from the transgene (Fig. 1). In this experiment, elastin was detected using the anti-human recombinant tropoelastin antibody, which has been shown to cross-react with the bovine protein. As expected, untransfected cells did not contain elastin in their extracellular matrix (Fig. 2A). Because earlier studies had suggested that the C terminus of tropoelastin contains a critical assembly site (16), our truncation and mutation constructions focused on this region. Exon 36 encodes a conserved sequence that contains the molecule’s only 2 Cys residues and a terminal Arg-Lys-Arg-Lys...
sequence. The cysteine residues have been shown to form a disulfide-bonded loop structure that creates a highly charged "pocket" at the end of the molecule believed to facilitate interactions between tropoelastin and highly acidic microfibrils. It was an antibody to this exon 36 sequence that disrupted elastin assembly in studies by Brown-Augsburger et al. (16). To assess the importance of this region of the protein to elastin assembly, we generated tropoelastin constructs in which first exon 36, protein from constructs lacking exons 36, was deleted (Fig. 2D). Using species-specific antibodies, it was possible to determine whether either of the transgenes was able to incorporate into elastic fibers made by the mouse and whether either acted through a dominant-negative mechanism to disrupt normal mouse elastic fiber assembly. The constructs consist of linearized forms of the transfection constructs used in the in vitro experiments. They contain the immediate-early components of the cytomegalovirus promoter to guide expression of the transgene and a SV40-derived 3' untranslated region and poly(A) signal.

After founder lines were stabilized, animals were tested for expression of the transgene. RT-PCR performed on RNA isolated from full-length and exon 1–28 transgenic animals showed that message from the transgene was being transcribed and was stable in these animals (lung tissue shown in Fig. 4). Using an antibody that detects human and bovine elastin, but not mouse elastin, and one that detects only mouse elastin, we assayed frozen sections from wild-type (non-transgenic) and transgenic lungs showing no staining with the bovine-specific antibody (Fig. 4A), but significant elastic fiber staining with the mouse antibody was observed (Fig. 4D). Analysis of the four founders expressing the full-length bovine tropoelastin transgene found uniform deposition of bovine elastic fibers in the heart and developing aorta (data not shown), consistent with past studies showing strong expression of the cytomegalovirus promoter in these tissues (21–23). In other tissues, deposition of bovine elastic fibers differed among founders, most likely resulting from transcriptional differences associated with positional effects surrounding the location of transgene insertion. Commonly positive tissues included lung (Fig. 4B), kidney, bladder, and small blood vessels of the liver (data not shown). In all tissues in which the full-length transgene was expressed in a given animal, the bovine protein was found to incorporate into existing mouse elastic fibers with no obvious alteration of fiber structure (Fig. 4E). In contrast to what was found with the full-
A synthetic peptide encoded by exon 30 was dissolved in water and allowed to form aggregates overnight. Precipitates were pelleted and prepared for quick-freeze deep-etch microscopy. The micrograph shows that the exon 30 peptides polymerized into fibers. Scale bar = 50 nm.

**Fig. 5.** Exon 30 peptide forms amyloid-like fibers. A synthetic peptide encoded by exon 30 was dissolved in water and allowed to form aggregates overnight. Precipitates were pelleted and prepared for quick-freeze deep-etch microscopy. The micrograph shows that the exon 30 peptides polymerized into fibers. Scale bar = 50 nm.

The quantity or quality of fibers formed (data not shown). No fibers were ever observed with the exon 30 scrambled peptide.

Deletion of Exon 30 Decreases, but Does Not Ablate, Elastic Fiber Formation—The propensity of the exon 30 peptide to form amyloid-like aggregates and the failure of tropoelastin truncation constructs lacking exon 30 to associate with the PE cell matrix suggested that the exon 30 sequence might facilitate deposition of tropoelastin into the extracellular matrix and hence influence elastin fibrillogenesis. This possibility was tested by expressing, in the PE cell system, a tropoelastin construct with only exon 30 deleted (Δ30). Relative to the full-length construct (Fig. 6A), deposition of the Δ30 protein into matrix fibers was greatly reduced (Fig. 6B). Rare fields were present, however, in which deposition of Δ30 did take place. Expression of a construct containing an internal deletion of a different hydrophobic exon in the C-terminal domain, exon 32 (Δ32), yielded an elastin product that was deposited into fibers in the extracellular space. Although the fibers may be qualitatively different from those produced by the full-length construct (Fig. 6C), the fact that they were deposited shows that the result found for Δ30 was not simply a consequence of exon deletion in general.

Expression of construct Δ30 as a transgene in mice gave results similar to those observed with PE cells. The Δ30 protein was detected in several tissues and organs of the transgenic mice, but the amount of incorporated protein was much less than what was found for the full-length transgene (Fig. 7).

Identification of a Hypersensitive Protease Site That May Influence Assembly—In analyzing tropoelastin secreted into the culture medium of transfected PE cells, we noticed several proteolytic breakdown products associated with the full-length molecule and mutation forms that were not evident in samples of the bTE-(1–28) protein (Fig. 3). Further repetitions of the experiment revealed varying degrees of degradation for all of the constructs from experiment to experiment. The only construct for which fragmentation was reproducibly decreased was bTE-(1–28). The molecular mass of the primary breakdown product of the full-length molecule was similar in size to the intact bTE-(1–28) protein (i.e., ~55 kDa), suggesting that a cleavage site may be present near the exon 28 border that, when cleaved, leads to removal of the C-terminal assembly sequence.

To investigate susceptible sites for proteolytic cleavage, we treated recombinant tropoelastin with plasmin for times ranging from 30 min to 2 h. Earlier studies determined that the proteolytic degradation of tropoelastin isolated from developing chick
transgenic animals expressing full-length bovine tropoelastin showed significant deposits of bovine tropoelastin, whereas the lungs (B) and hearts (D) from Δ30 animals revealed only minimal staining.

aorta could be largely prevented by the inclusion of inhibitors of trypsin-like proteases (28, 29). Given that plasminogen, the pro form of the enzyme plasmin, is a trypsin-like protease present in high concentrations in serum and that blood vessels are a key location for elastin assembly, the choice of this protease seemed to be a reasonable one. When recombinant tropoelastin was treated with plasmin at 37 °C, a ladder of fragments similar to the naturally occurring breakdown products was detected (data not shown). At early time points, many fragments of various sizes were present, but by 2 h of treatment, only one resistant C-terminal fragment remained (Fig. 8), as evidenced by Western blotting of the proteolytic fragments probed with an antibody specific for the extreme C terminus of tropoelastin (CTe) (16). Our initial mutation and deletion constructs expressed in PE cells were focused on exon 36, but we found that these amino acids are not required for elastin secretion or for its deposition into the extracellular matrix. In fact, mutation of one or both of the cysteine residues to alanine, deletion of the terminal RRKRK sequence, or deletion of the entire exon 36 had no observable effect on the ability of the mutant protein to associate with microfibrils. Rather, a large portion of the C-terminal region consisting of exons 29–36 had to be deleted before elastin accumulation in the extracellular matrix could be entirely inhibited. Because the truncated molecules were effectively secreted and were stable in the culture medium, these findings suggest that sequence 29–36 contains epitopes, exclusive of exon 36, necessary for mediating the association of tropoelastin with microfibrils in the extracellular matrix.

Although the PE cell assay provides an excellent means to assess whether a given form of tropoelastin can associate with microfibrils in the extracellular matrix, its reliance on immunofluorescence co-localization provides only limited information about the quality of the resultant elastic fiber. We were not able to determine by this method, for example, whether any of the deletion constructs found to co-localize with microfibrils associated less efficiently than the full-length protein or bound in a way that precluded later assembly steps. However, that there may be quantitative differences is suggested by preliminary experiments in which desmosine levels were found to be lower in constructs lacking exon 36. This result is consistent

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**FIG. 6.** Deletion of exon 30 decreases, but does not completely inhibit, elastin deposition. PE cells transfected with the full-length (A) or Δ30 (B) expression construct were stained with an antibody to detect bovine tropoelastin. Deposition of the Δ30 protein into the extracellular matrix was significantly reduced relative to the full-length protein. A construct containing an internal deletion of exon 33 (Δ33), another hydrophobic exon in the C-terminal domain, yielded an elastic matrix similar in appearance to that generated by the full-length construct (C).

**FIG. 7.** Immunostaining of bovine tropoelastin in full-length and Δ30 transgenic animals. The lungs (A) and hearts (C) from transgenic animals expressing full-length bovine tropoelastin showed significant deposits of bovine tropoelastin, whereas the lungs (B) and hearts (D) from Δ30 animals revealed only minimal staining.

**FIG. 8.** Bovine tropoelastin contains a plasmin-resistant C-terminal fragment. Shown are Western blots of recombinant bovine tropoelastin cleaved with plasmin for 0, 30, 60, and 120 min at 37 °C. The blots were probed with antibody CTe, which is reactive against exon 36 and therefore detects fragments with an intact C terminus. Multiple fragments were initially generated by cleavage with plasmin; but by 2 h, only a single C-terminal fragment remained.
with data reported by Hsiao et al. (30) showing decreased cross-linked elastin in the matrices of cells to which tropoelastin lacking exon 36 had been added.

The importance of the C-terminal sequence to tropoelastin assembly was confirmed using transgenic mice, which also allowed us to determine whether tropoelastin molecules bearing C-terminal mutations would lead to defects in elastic tissues through dominant-negative effects. Characterization of the different mouse lines showed that animals expressing wild-type bovine tropoelastin transgenes successfully incorporated the protein into the mouse elastic fiber. In contrast, no bTE(1–28) protein could be detected in any of the tissues in which mRNA for the transgene was readily identified. Incorporation of a transgenic construct lacking exon 30 into elastic fibers was observed, but at levels significantly lower than those of the full-length protein. Each of these findings is consistent with results found in PE cells, where construct Δ30 was found to associate with microfibrils at low levels relative to the full-length transgene product, and construct 1–28 was found only in the culture medium. Because phenotypic and histopathological assessment of all the transgenic lines found no adverse effects of transgene expression, our results demonstrate that incorporation of the full-length bovine protein into the mouse fiber did not disrupt the assembly or function of the endogenous mouse elastin. Similarly, expression of assembly-incompetent forms of the protein (1–28 and Δ30) did not interfere in a dominant-negative fashion with deposition of normal elastin. Similar results were obtained by Sechler et al. (31), who showed that transgenic mice expressing rat constructs (full-length and the naturally occurring splice variant Δ13–15) as transgenics yield healthy animals with no observable harmful health effects.

Fine mapping of the matrix-binding activity of tropoelastin using synthetic peptides or expression constructs with single exon deletions localized sequences in and around exon 30 as being the major interactive site. The sequence encoded by exon 30 contains a tandem repeat (GGLG(V/A)) that resembles sequences found in other proteins that aggregate via β-sheet/β-turn structures. Examples include lamprin (a matrix protein of the lamprey annular cartilage) (24), spidrin (a spider dragline silk protein) (32), and various matrix proteins of the chorion or the eggshell membrane of insects (33–35). These proteins assemble through the interdigitation of side chains belonging to residues present in short stretches of cross-β-conformation (β-turn/β-sheet) (25, 36). Based on these structural similarities, Robson et al. (24) have suggested that sequences of this type contribute to self-aggregation of elastin monomers and alignment of polypeptide chains for cross-linking. Results presented in this report confirm that exon 30 does indeed contribute to tropoelastin assembly, but most likely in the context of interaction with microfibrils. Previous studies in our laboratory have demonstrated interactions between tropoelastin and small expression constructs containing the Pro- and Gly-rich regions of fibrillin-1 and fibrillin-2, respectively (37). Although we did identify the amino acid sequence responsible for tropoelastin binding in the fibrillin fragments, it is interesting to note that the glycine-rich region of fibrillin-2 contains several repeats of the GXXGXX sequence that could interact with exon 30 of tropoelastin via β-sheet/β-turn structures.

The presence of an assembly site centered on exon 30 has important implications for understanding both normal elastin assembly and the molecular mechanisms of diseases arising from mutations within the elastin gene. For example, the characteristic mutations reported for autosomal dominant cutis laxa are single nucleotide deletions in exons 30 and 32 that result in out-of-frame sequence extending into the 3′-untranslated region with notable loss of the cysteine residues in exon 36. It has been speculated that this abnormal sequence might disrupt assembly of normal elastin in a dominant-negative fashion. In contrast, the majority of mutations in isolated SVAS are point mutations that produce premature termination sites. Urbán et al. (13, 38, 39) have shown that many of these mutations produce an unstable mRNA transcript that is rapidly degraded through nonsense-mediated decay, resulting in elastin haploinsufficiency at the RNA level. In one case, however, mRNA and protein from a mutant elastin gene were identified in cells from an SVAS individual, although at reduced levels compared with the wild-type allele (13). If mRNA from a gene with a truncation mutation escapes nonsense-mediated decay, the absence of a C-terminal assembly domain may preclude its incorporation into the growing fiber. The result would be haploinsufficiency at the protein level.

Removal of the C-terminal domain by proteolytic events outside the cell also appears to be a mechanism for regulating elastin assembly in instances of normal tissue growth and remodeling. For example, closure of the ductus arteriosis shortly after birth involves dissolution of the vessel's elastic laminae and ingrowth of intimal cushions. At the same time, tropoelastin secreted by ductus arteriosis smooth muscle cells is inhibited from forming new elastic fibers by proteolytic removal of the C-terminal domain (40). Although the exact cleavage site within the tropoelastin molecule was not characterized in the ductus arteriosis study, the size of the truncated protein (52 kDa) is similar to that of the fragment expected when tropoelastin is cleaved with plasmin at exon 26. This site in exon 26 has previously been described as being susceptible to cleavage by kallikrein and thrombin (41) and clearly defines a location on the surface of the tropoelastin molecule that is accessible to trypsin-like proteases. The identification of a hypersensitive protease site and the finding in the ductus arteriosis study that the C-terminal region of tropoelastin can be specifically removed by proteolysis provide evidence for a possible mechanism for extracellular control of elastic fiber formation.

It has long been assumed that coacervation of tropoelastin is a crucial step in assembly of the elastic fiber (42–44). Our data suggest, however, that coacervation may not be involved in the initial steps of elastin assembly and that coacervation by itself cannot drive elastin assembly in tissues. Coacervation, an entropically driven, inverse temperature transition caused by the interaction of the hydrophobic domains in the molecule, occurs as tropoelastin monomers associate to form large aggregates. Several laboratories have shown that the large hydrophobic sequences in the middle of the molecule play a dominant role in the intermolecular interactions that occur during coacervation (43). If coacervation were the only requirement for assembly, then all of our deletion constructs would form fibers (or at least aggregates) because they all contain the critical middle hydrophobic sequences. This was clearly not the case, as constructs bTE(1–28) and Δ30 did not undergo assembly when expressed in either PE cells or transgenic mice. Instead, our results argue for a model of nucleated assembly in which the tropoelastin monomer interacts with microfibrils in a process mediated by the exon 30 assembly domain. This process is initiated by β-structure interactions between exon 30 of tropoelastin and β-structure-containing regions on microfibrillar proteins such as the glycine-rich portion of fibrillin-2. Consequently, we believe that microfibrils are required to initiate or greatly enhance the rate of the assembly process through an interaction with the C-terminal region of tropoelastin. We cannot rule out a role for coacervation in directing tropoelastin self-interaction in later stages of fiber assembly; however, we do not know at what point microfibrils are no longer required.
Acknowledgments—We thank Clarina Tisdale for technical assistance in the generation of antibodies and cutting of frozen sections, Chris Ciliberto for maintenance and genotyping of transgenic animals, and Tom Broekelmann for additional technical support. We also acknowledge Ron McCarthy and the Program in Lung Biology at the Washington University School of Medicine for the production of the transgenic animals used in this study.

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