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Modification and Functional Inactivation of the Tropoelastin Carboxy-terminal Domain in Cross-linked Elastin

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Abstract

The carboxyl terminus of tropoelastin is a highly conserved, atypical region of the molecule with sequences that define both cell and matrix interactions. This domain also plays a critical but unknown role in the assembly and crosslinking of tropoelastin during elastic fiber maturation. Using a competitive ELISA with an antibody to an elastase-resistant epitope in the carboxy-terminus of tropoelastin (domain-36), we quantified levels of the domain-36 sequence in elastase-derived peptides from mature, insoluble elastin. We found that the amount of carboxy-terminal epitope in elastin is ~0.2% of the expected value, assuming each tropoelastin monomer that is incorporated into the insoluble polymer has an intact carboxy-terminus. The low levels suggest that the majority of domain-36 sequence is either removed at some stage of elastin assembly or that the antigenic epitope is altered by posttranslational modification. Biochemical evidence is presented for a potential lysine-derived cross-link in this region, which would alter the extractability and antigenicity of the carboxy-terminal epitope. These results show that there is little or no unmodified domain-36 in mature elastin, indicating that the cell and matrix binding activities associated with this region of tropoelastin are lost or modified as elastin matures. A crosslinking function for domain-36 may serve to help register the multiple crosslinking sites in elastin and explains why mutations that alter the domain-36 sequence have detrimental effects on elastic fiber assembly.

Keywords

elastin; elastic fiber assembly; tropoelastin; exon-36; elastin diseases

1. Introduction

Elastin is the major extracellular matrix protein capable of elastic recoil in tissues subjected to repeating cycles of extension and contraction; such as the aorta, skin, and lung. The secreted form of elastin (tropoelastin) is a protein comprised of alternating hydrophobic and lysine rich sequences (Bressan et al., 1987; Mithieux and Weiss, 2005). All but ~10% of the 35-40 (depending on species) lysine residues are modified to form unique bifunctional or tetrafunctional cross-links that covalently link monomeric tropoelastin molecules into the mature, functional polymer (Franzblau et al., 1969; Gerber and Anwar, 1974). The mature

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protein is insoluble, hydrophobic in character, resistant to most proteases, and has a very low turnover rate (Partridge, 1962; Shapiro et al., 1991).

How tropoelastin monomers assemble prior to crosslinking is still not completely understood. Like collagen, tropoelastin monomers are capable of self-association but proper elastic fiber assembly requires the participation of both cells and ancillary proteins. Functional mapping studies identified domains in the C-terminal half of tropoelastin that facilitate fiber assembly. Tropoelastin lacking the sequence in domain[1] 30, for example, does not interact with microfibrils in the extracellular matrix and, hence, does not assemble into fibers (Kozel et al., 2003). Mutations in the elastin gene that delete this region of the protein are associated with supravalvular aortic stenosis, an autosomal dominant disease arising from elastin haploinsufficiency (Li et al., 1997;Urban et al., 2000). A second important assembly site is located at the C-terminus of the molecule and is encoded by exon 36, the last exon in the gene. Antibodies directed to this domain prevent elastin assembly (Brown-Augsburger et al., 1996) and deletion of this sequence leads to a dramatic reduction in levels of cross-linked elastin in *in vitro* culture systems (Hsiao et al., 1999). This region of the protein is highly conserved (Chung et al., 2006) and interacts with microfibrillar proteins (Brown-Augsburger et al., 1994), integrins (Rodgers and Weiss, 2004) and sulfated proteoglycans (Broekelmann et al., 2005). An autosomal dominant form of cutis laxa has been linked to mutations that alter the sequence of this region of the molecule (Szabo et al., 2006;Zhang et al., 1999).

The sequence encoded by domain-36 is unique; it is not a typical crosslinking or hydrophobic domain, but it does contain a hydrophobic cluster at the N-terminal end of the sequence as well as a cluster of positively charged amino acids (usually RKRK) at the C-terminus. This sequence also contains the protein's only two cysteine residues, which are disulfide bonded to form an intrachain loop structure (Brown et al., 1992; Floquet et al., 2005). How this region of the protein facilitates elastin assembly is still not known. Based on pulse-chase studies of tropoelastin production/degradation by rat aortic smooth muscle cells, it was proposed that the C-terminal region is important in directing fiber assembly similar to the role played by extension peptides of the fibrillar collagens (Chipman et al., 1985; Franzblau et al., 1989). Pulse-chase data suggested that after facilitating incorporation of the tropoelastin molecule into the growing elastic fiber, the C-terminal “pro” peptide is removed by a specific tryptic-like protease and is not incorporated into the mature elastic structure. Other investigators, however, have identified C-terminal sequences in solubilized peptides from insoluble elastin using specific antibodies (Rosenbloom et al., 1986) or by mass spectrometry (Getie et al., 2005; Schmelzer et al., 2005), implying that the C-terminus may not be processed off during fiber assembly.

The status of the carboxyl terminus in mature elastin is important. Its presence or absence in the mature protein will provide important clues as to its role in fiber assembly. Furthermore, as others and we have shown, the C-terminal sequence can interact with adhesive receptors on cells and, in this capacity, could provide a substrate for cellular adhesion to elastic fibers. Alternatively, the sequence could serve a signaling function, particularly if liberated from the insoluble polymer by proteolytic events associated with inflammation or tissue remodeling. Indeed, elastin peptides have been shown to recruit inflammatory cells to regions of damage in the lung (Houghton et al., 2006) and are responsible for progression of vascular injury in animal models of aneurysmal disease (Hance et al., 2002).

In this report, we use a domain-36-specific antibody to evaluate the presence or absence of the C-terminal sequence in mature elastin. We show that the sequence recognized by the antibody

[1]Domain numbers refer to sequences encoded by individual exons in the elastin gene.

is present but underrepresented in mature elastin, and modified most likely due to crosslinking of one or more of the lysine residues.

2. Results

2.1 Antibody Specificity for C-terminal Sequence

A key reagent in our identification and quantification of the C-terminal domain in elastin is an antibody generated to a synthetic peptide containing a sequence encoded by exon 36 of bovine tropoelastin (anti-CTP) (Brown-Augsburger et al., 1996). This antibody blocks the association of tropoelastin with microfibrils and decreases the amount of cross-linked elastin when added to cells *in vitro* (Brown-Augsburger et al., 1996). Hence, the antibody recognizes an epitope important for elastic fiber assembly. To show that the antibody is specific for elastin and only for the C-terminal sequence to which it was generated, we undertook an extensive characterization of specificity. Figure 1 is an immunoblot showing that the CTP antibody reacts with a single band of appropriate size for tropoelastin (~65 kDa) in a cell extract of FBAF cells (Panel A lane 1). Reactivity of the antibody to this band is completely blocked by pre-incubation with the synthetic peptide to which the antibody was produced (Panel A lane 2).

Western blot analysis using bacterially produced 6-His tagged and purified fragments of tropoelastin shows reactivity only with molecules that contain domain-36 at the C-terminus. The first panel in Figure 1B is a Coomassie-blue stained gel confirming that approximately equivalent amounts of purified protein were loaded for each fragment. The second panel is a western blot using the CTP antibody and shows reactivity to full-length tropoelastin (domains 1-36), domains 15-36 and domains 29-36, but no reactivity to fragments lacking the C-terminus (domains 1-15 and 15-29). The third panel is the negative control for the immunoblot using the CTP antibody blocked by pre-incubation with the immunizing peptide.

Figure 2A shows a series of ELISA dilution curves for the CTP antibody on plates coated with different bacterially expressed tropoelastin fragments. The half maximal reactivity for this antibody is approximately 160 μg/ml for substrates coated with full-length tropoelastin and 80 μg/ml for substrates coated with either the domain 15-36 or the domain 29-36 fragments. The antibody does not react with substrates coated with domain 1-15 or domain 15-29 fragments. Similar results are obtained when the ELISA plates are coated with synthetic peptides containing sequences at the C-terminus of tropoelastin (Figure 2B). Half maximal reactivity occurs at approximately 80 μg/ml for a substrate coated with the CT-25 peptide and at 100 μg/ml for a substrate coated with the CT-14 peptide. There is slight antibody reactivity towards the e35 peptide and minimal reactivity towards a scrambled e35-36s peptide. Together, the western blot and ELISA assays show that the CTP antibody is specific for the C-terminal sequence.

2.2 Epitope mapping

Fine mapping of the anti-CTP epitope was achieved using a competitive immunoassay comparing competition between tropoelastin and various synthetic peptides (Table I) containing permutations of the C-terminal sequence. As expected, soluble tropoelastin blocks anti-CTP binding to a tropoelastin-coated substrate (Figure 3A). Similarly, the CT-25 peptide in solution fully competes, at the same molar concentration as tropoelastin, for anti-CTP binding to the tropoelastin substrate. Using a substrate coated with the CT-25 peptide, soluble CT-25 fully blocked anti-CTP binding whereas the CT-14 peptide blocked binding by ~75% (Figure 3B). When the substrate was coated with the CT-14 peptide, both CT-25 and CT-14 peptides fully blocked anti-CTP binding (data not shown). This confirms that the terminal 25 residues contain all the major epitopes for the anti-CTP antibody. The major epitopes are in the terminal 14 residues (encoded by exon 36), but there is a minor contribution by the 11

residues at the N-terminus of CT-25 that are contributed by exon 35 (exon 33 in human). The control scrambled e36-36s peptide failed to inhibit anti-CTP binding to a CT-25 coated plate.

2.3 Protease sensitivity of anti-CTP epitope

The susceptibility of the CTP epitope in tropoelastin to elastase treatment was determined by comparing the antigenic reactivity of elastase-digested tropoelastin with tropoelastin not exposed to protease. Elastase digestion did not decrease the reactivity of tropoelastin to the CTP antibody even through the protein was digested to small fragments (Figure 4A).

The stability of the CTP epitope was further characterized by assessing the effects of pancreatic elastase, pepsin, and trypsin on CT-25 peptide (Figure 4B). Anti-CTP immunoreactivity was eliminated from the peptide by a 15-minute exposure to trypsin and reduced by half after prolonged pepsin treatment. Treatment with elastase, however, had no effect on the immunological properties of the CT-25 peptide, even though it was cleaved by elastase at multiple sites (Figure 5). Characterization of the elastase-derived fragments by HPLC and mass spectrometry identified cleavage sites on either side of the alanine at position 7, N-terminal to the glycine at position 9, and C-terminal to the alanine at position 14. These findings show that elastase can be used to solubilize insoluble elastin without compromising the reactivity of the C-terminal epitope for the CTP antibody.

2.4 CTP in insoluble elastin

The stability of the anti-CTP epitope makes it possible to quantify the amount of CTP in elastin following solubilization by elastase. Figure 6A shows the amount of elastin solubilized from 50 mg neutral salt purified elastin (~770 nmoles using 65,000Da for the molecular mass of tropoelastin) by different concentrations of pancreatic elastase. Soluble protein concentrations were determined by amino acid analysis of the reaction supernatant. A 500μg dose of elastase visibly solubilized all of the elastin in this assay, and increasing the dose to 1mg elastase failed to increase the amount of solubilized elastin as determined by amino acid analysis or competitive ELISA. This confirms that elastin was completely solubilized at elastase levels of 500 μg and higher. Complete solubilization was also confirmed by recovery of desmosine in the reaction supernatant (120 ng desmosine/mg protein, which is equivalent to desmosine levels measured in insoluble elastin prior to enzymatic digestion).

Figure 6B shows immunoreactive CTP in the same sample as determined by competitive ELISA. Liberation of the CTP epitope by elastase followed the same kinetics observed for solubilization of elastin based on total protein (Figure 6A). However, the total amount of CTP measured by competitive ELISA is less than what would be expected based on the total amount of elastin. Our recovery of ~1.5 nmoles of CTP epitope is well below the expected value of 770 nmoles that is assumed if each tropoelastin monomer incorporated into the insoluble polymer has an intact C-terminus. Expressed as a percentage, the amount of immunologically reactive C-terminus is ~0.2% of the expected value, which corresponds to one CTP epitope per 455 molecules.

2.5 Relative content of the C-terminus in elastin isolated using different procedures

To determine if the relatively harsh procedures typically used to purify insoluble elastin contribute to the low CTP levels, we assessed the amount of anti-CTP reactivity in elastase digests of several common elastin preparations as well as in crude ligamentum nuchae. Figure 7 shows that the amount of CTP expressed as a percent of total elastin protein is low (0.1-0.3%) for all samples. This includes crude ligamentum nuchae, which has not undergone extraction. Interestingly, no detectable CTP epitope was evident in elastase digests of elastin purified by hot base hydrolysis (Lowry et al., 1941). This result is not unexpected since hot NaOH is known

to introduce extensive peptide bond cleavage and protein degradation into the purified polymer (Daamen et al., 2001;Soskel and Sandburg, 1983;Starcher and Galione, 1976).

2.6 Remaining CTP epitope in insoluble elastin is trypsin insensitive

The Starcher method for elastin isolation begins with elastin purified by neutral salt extraction and then uses trypsin and CNBr digestion followed by extraction with urea and dithiothreitol (Starcher and Galione, 1976). The results in Figure 7 indicate that Starcher elastin contains the same amount of CTP epitope as neutral salt elastin, even though our studies with CT-25 show that trypsin digestion is capable of destroying the CTP epitope. To verify that the CTP antibody-reactive peptides released from insoluble elastin by elastase are resistant to trypsin treatment, neutral salt elastin was solubilized by elastase digestion and fractionated by reverse phase HPLC. Fractions with reactivity toward CTP antibody were pooled, treated with trypsin, and assayed for immunoreactivity by ELISA. Figure 8 confirms that the small amounts of CTP epitope obtained from mature elastin is not destroyed by trypsin digestion.

3. Discussion

Assessing the presence or absence of the C-terminal sequence in insoluble elastin has been problematic. It was originally proposed that this region served a pro-peptide function, similar to the pro-peptides of collagen, and was removed concurrent with assembly (Chipman et al., 1985; Franzblau et al., 1989). Other evidence, however, suggested that it is retained in the crosslinked polymer. Several biochemical studies have tried to use the cysteine residues unique to this region as markers during peptide purification (Price et al., 1993), but contaminants from cysteine-rich microfibrils make this approach difficult. Another complicating factor is suggested by our finding that purification of elastin using hot NaOH modifies or removes the C-terminal sequence. Thus, the nature of the starting material one chooses to study is perhaps more important than previously appreciated. Our findings described in this report suggest that the domain-36 sequence is, in one form or another, present in mature elastin that has not be prepared using NaOH. However, the low levels that we detect using a quantitative immunological approach suggest that the majority of domain-36 sequence is either removed at some stage of elastin assembly or that the antigenic epitope is altered by posttranslational modification. It is also important to note that low levels of CTP were detected in unprocessed ligamentum nuchae, a tissue highly enriched in elastin. This suggests that CTP modification occurs coincident with the incorporation of tropoelastin into the mature polymer. As mentioned above, domain-36 of tropoelastin has properties that are important for the biological activity of the molecule. The results shown here indicate that there is little or no intact, unmodified domain-36 in mature elastin. Thus, the cell and matrix interaction properties found in this region of tropoelastin are lost as elastin matures.

The quantitative recovery of CTP activity when tropoelastin or CT-25 is treated with elastase (Figure 4) confirms that the estimate of CTP levels in insoluble elastin are not artifactually low due to inactivation of the antigenic epitope by elastase digestion. The question then becomes whether something other than removal of the CTP during assembly can explain the low levels. The presence of the CTP epitope in Starcher-purified elastin is particularly informative in this regard. Starcher elastin, which utilizes trypsin treatment in the purification protocol, contains the same amount of CTP epitope as neutral salt purified elastin even though trypsin digestion is capable of destroying the CTP epitope. Resistance to trypsin digestion suggests that one or more of the lysine residues normally recognized by trypsin is modified in the domain-36 sequence once tropoelastin is incorporate into the insoluble polymer. The most likely modification is crosslinking to another lysine residue, which is supported by studies of Mithieux et al (2005) who demonstrated that lysines in the RKRK (domain-36) sequence of purified tropoelastin are particularly sensitive to lysyl oxidase modification and are capable of

forming a bifunctional cross-link with lysine elsewhere in the molecule. A high crosslinking frequency associated with lysines in domain-36 was also demonstrated using bifunctional crosslinkers (Wise et al., 2005). Lysine alteration through cross-link formation would likely alter the antigenicity of the CTP epitope and covalently lock the CTP into the mature polymer. Hence, epitope modification and low extractability would explain the low levels of CTP reactivity found in the elastase digests. It is also possible that the C-terminal region is removed from some molecules during assembly, while that which remains participates in a crosslink.

Our studies are similar to those of Rosenbloom et al. (1986) who, using an antibody generated to a similar C-terminal peptide, found reactivity in neutrophil elastase digests of insoluble elastin. Other than demonstrating the presence of the C-terminal epitope amongst the solubilized peptides, the amount of recovered peptide was not quantified. It is interesting to note that these authors identified CTP reactivity in digests of NaOH-treated elastin whereas we did not. Nevertheless, both studies support the presence of C-terminal sequences in insoluble elastin. Also consistent with retention of the CTP in mature elastin are mass spectrometry studies that have identified fragments of the CTP in proteolytic digests. Getie et al. (2005), for example, identified a peptide in an elastase digest of skin elastin that contains the residues N-terminal to the first cysteine in domain-36, which is consistent with our finding of an elastase cleavage site at this sequence (Figure 5). An analysis of a thermolysin digest of the same elastin identified sequence immediately upstream of RKRK, including the two cysteine residues (Schmelzer et al., 2005). In neither study, however, were the lysine residues in domain-36 identified, which is consistent with their modification to form cross-links.

The presence of a cross-link in domain-36 suggests how this domain may play a critical role in elastin assembly. Expression of tropoelastin deletion constructs *in vitro* and as transgenes in mice have identified sequences encoded by exon 30 as being critical for the self-association of tropoelastin molecules (Kozel et al., 2003). Tropoelastin constructs lacking domain-36, however, were able to associate with existing elastic fibers, but crosslinking was greatly attenuated (Hsiao et al., 1999; Kozel et al., 2003; Sato et al., 2007). These studies support a multistep process for elastin assembly that involves tropoelastin self-association (mediated by domain 30) followed by crosslinking and maturation mediated by domain-36 (Czirok et al., 2006; Kozel et al., 2004; Kozel et al., 2006; Sato et al., 2007). The data from the current study are consistent with this model and suggest that domain-36 may function to facilitate fiber maturation by forming an initial cross-link that serves to help register the multiple tropoelastin crosslinking sites for efficient oxidation by lysyl oxidase. A crosslinking function would explain why mutations that alter the domain-36 sequence, such as frame shift mutations associated with dominant cutis laxa (Szabo et al., 2006; Zhang et al., 1999), have detrimental effects on elastic fiber assembly.

4. Experimental Procedures

4.1 Insoluble elastin and synthetic peptides

Crude defatted bovine ligamentum nuchae and bovine insoluble elastin prepared using the Lansing (Lansing et al., 1951), Starcher (Starcher and Galione, 1976), and neutral salt extraction techniques were obtained from Elastin Products Company. All peptides were synthesized using an ABI-431A synthesizer with FastMoc chemistry on Wang-capped resins. After cleavage, peptides were dissolved in MilliQ water with 0.05% trifluoroacetic acid and purified with reverse phase HPLC[2] on a Vydak 218TP1022 C-18 column using a linear 0-50% acetonitrile gradient. Peak fractions were dried by rotary evaporation, dissolved in MilliQ water

[2]Abbreviations: ABTS, 2,2'-azino-di-(3-ethylbenzthiaaoline-6-sulfonate; FBAF, fetal bovine adventitial fibroblasts; CTP, carboxyl terminal peptide; HPLC, high pressure liquid chromatography; AAA, amino acid analysis; ELISA, enzyme linked immunosorbent assay; PPE, porcine pancreatic elastase; PMSF, phenylmethylsulfonylfluoride.

then lyophilized. Peptides containing cysteine were dissolved in water and disulfide bonds were allowed to form before use (Brown-Augsburger et al., 1996). LC-mass spectrometry performed by the Protein and Nucleic Acid Chemistry Laboratory at Washington University Medical School verified the identity of each purified peptide. Protein and synthetic peptide concentrations were determined by amino acid analysis using a Beckmann 6300 high performance amino acid analyzer. Synthetic peptide sequences are listed in Table I. The CTP peptide was used to generate the anti-CTP antibody and is the C-terminal 19 residues of elastin, CT-25 includes the C-terminal 25 residues, and the CT-14 peptide includes the C-terminal 14 residues of elastin. The e35 peptide includes 11 residues from domain 35, and the e35-36s peptide is a scrambled peptide from domain 35-36 sequences.

4.2 SDS-PAGE, antibodies and western blots

Anti-CTP (Brown-Augsburger et al., 1996) is a rabbit polyclonal raised against a synthetic peptide from the C terminus of elastin (CTP, Table I) and purified by the caprylic acid method of Steinbuch (Steinbuch and Audran, 1969). SDS-PAGE and Western blot analyses were performed as described (Brown-Augsburger et al., 1996) using enhanced chemiluminescent reagents (ECL from GE Healthcare) to detect horseradish peroxidase conjugated secondary antibodies (donkey anti-rabbit horseradish peroxidase from GE Healthcare).

4.3 Expression and purification of tropoelastin and fragments

Tropoelastin and tropoelastin fragments were expressed as 6-His fusion proteins using the pQE expression system (Qiagen, Inc.) in the M-15 strain of *E. coli* (Broekelmann et al., 2005). Expressed proteins were initially purified using nickel chromatography under denaturing conditions with a batch purification method recommended by the manufacturer. Eluted tropoelastin or tropoelastin fragments were dialyzed against 50mM acetic acid and lyophilized. The proteins were further purified using reverse phase HPLC chromatography on a Vydak 214TP510 C-4 column using a 0-50% linear acetonitrile gradient.

4.4 Cells

Fetal bovine adventitial fibroblasts (FBAF) were obtained from the explanted adventitial layer dissected from the aorta of 170-day gestation fetal calf (Wrenn et al., 1986). Cells were maintained in DMEM + 20% fetal calf serum (Biomeda) and subcultured 1:3 weekly.

4.5 Direct ELISA

EIA plates (96 well, Corning) were coated with various protein fractions at a concentration of 25μg/ml in 0.01M sodium carbonate pH=9.6. The plates were washed and blocked with PBS containing 1% nonfat dry milk, 0.1% fish gelatin (Sigma chemical G-7765), and 0.1% TWEEN 20 (PBS-milk). Dilutions of anti-CTP were added for 1 hour at room temperature, non-bound antibodies were removed and bound antibodies detected with secondary donkey anti-rabbit IgG-horseradish peroxidase conjugates (GE Healthcare). The amount of secondary conjugate bound was determined with the peroxidase substrate 2,2′-azino-di-(3-ethylbenzthiaaoline-6-sulfonate (ABTS) (KPL Labs) at 410nm.

4.6 Competitive ELISA

EIA plates were coated with 150nM CT-25 peptide (Table 1) or 25μg/ml tropoelastin in 0.01M sodium carbonate pH=9.6. The plates were washed and blocked with PBS-milk. The soluble competitor (diluted in PBS-milk) and 100μg/ml (final concentration) anti-CTP were added to the plate. After one hour, the amount of bound anti-CTP was quantified as above. Known concentrations of soluble CT-25 were used to generate a standard curve.

4.7 Protease digestions

Bovine pancreatic trypsin (TPCK treated, Fluka) and porcine pancreatic elastase (PPE) (EC134, Elastin Products Company) were added to substrate at 1:100 enzyme to substrate ratio (w:w) unless otherwise indicated in 10mM HEPES, 1mM $CaCl_2$ pH=7.4. The reactions were stopped with the addition of phenylmethylsulfonylfluoride (PMSF) to 1mM. Pepsin (Sigma) digestion was performed in 10mM acetic acid at an enzyme to substrate ratio of 1:100 (w:w). The reaction was stopped by the addition of 1/10 volume of 1M Tris pH=8.8. HPLC purification of CT-25 fragments generated by PPE digestion was performed using reverse phase chromatography with a 4.6 × 25 cm C18 column (Beckman Ultrasphere ODS 235320) using a 0-50% acetonitrile gradient.

To determine the trypsin sensitivity of anti-CTP reactive fragments from insoluble elastin, 0.5 g neutral salt purified elastin was digested with 10mg PPE for 1 hour at 37°C. Soluble fragments were purified with reverse phase HPLC chromatography on a Vydak 218TP1022 C-18 column using a linear 0-50% acetonitrile gradient. Fractions immunoreactive with the CTP antibody were pooled, dried by rotary evaporation, and total protein determined by amino acid analysis. 10 mg of the sample was then digested for 1 hour at 37°C with 0.1mg trypsin. A control sample was treated the same but the trypsin was omitted. The reaction was stopped with the addition of PMSF to 1mM. The trypsin digested elastin or control elastin fragments were initially diluted to 1 mg/ml in 0.01M carbonate buffer then serially diluted and coated onto an ELISA plate. After blocking and washing, 100 μl of anti-CTP at 100 μg/ml was added for 1 hour at room temperature. Non-bound antibodies were removed and bound antibodies were detected with secondary donkey anti-rabbit IgG-horseradish peroxidase conjugates (GE Healthcare). The amount of secondary conjugate bound was determined colorimetrically with the peroxidase substrate ABTS at 410nm. The positive control for trypsin activity was the loss of the anti-CTP epitope in the CT-25 peptide.

4.8 Desmosine and isodesmosine determination

Elastin content in crude ligament or purified elastin was calculated from desmosine and isodesmosine levels determined by amino acid analysis using a Beckman 6300 high performance amino acid analyzer (Brown-Augsburger et al., 1995).

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References

Bressan GM, Argos P, Stanley KK. Repeating structures of chick tropoelastin revealed by complementary DNA cloning. Biochemistry 1987;26:1497–1503. [PubMed: 3593675]

Broekelmann TJ, Kozel BA, Ishibashi H, Werneck CC, Keeley FW, Zhang L, Mecham RP. Tropoelastin interacts with cell-surface glycosaminoglycans via its C-terminal domain. J. Biol. Chem 2005;280:40939–40947. [PubMed: 16192266]

Brown-Augsburger P, Broekelmann T, Mecham L, Mercer R, Gibson MA, Cleary EG, Abrams WR, Rosenbloom J, Mecham RP. Microfibril-associated glycoprotein (MAGP) binds to the carboxy-terminal domain of tropoelastin and is a substrate for transglutaminase. J. Biol. Chem 1994;269:28443–28449. [PubMed: 7961786]

Brown-Augsburger P, Tisdale C, Broekelmann T, Sloan C, Mecham RP. Identification of an elastin cross-linking domain that joins three peptide chains: Possible role in nucleated assembly. J. Biol. Chem 1995;270:17778–17783. [PubMed: 7629078]

Brown-Augsburger PB, Broekelmann T, Rosenbloom J, Mecham RP. Functional domains on elastin and MAGP involved in elastic fiber assembly. Biochem. J 1996;318:149–155. [PubMed: 8761465]

Brown PL, Mecham L, Tisdale C, Mecham RP. The cysteine residues in the carboxy-terminal domain of tropoelastin form an intrachain disulfide bond that stabilizes a loop structure and positively charged pocket. Biochem. Biophys. Res. Commun 1992;186:549–555. [PubMed: 1632791]

Chipman SD, Faris B, Barone LM, Pratt CA, Franzblau C. Processing of soluble elastin in cultured neonatal rat smooth muscle cells. J. Biol. Chem 1985;260:12780–12785. [PubMed: 4044609]

Chung MI, Ming M, Stahl RJ, Chan E, Parkinson J, Keeley FW. Sequences and domain structures of mammalian, avian, amphibian and teleost tropoelastins: Clues to the evolutionary history of elastins. Matrix Biol 2006;25:492–504. [PubMed: 16982180]

Czirok A, Zach J, Kozel BA, Mecham RP, Davis EC, Rongish BJ. Elastic fiber macro-assembly is a hierarchical, cell motion-mediated process. J. Cell. Physiol 2006;207:97–106. [PubMed: 16331676]

Daamen WF, Hafmans T, Veerkamp JH, Van Kuppevelt TH. Comparison of five procedures for the purification of insoluble elastin. Biomaterials Biomaterials 2001;22:1997–2005.

Floquet N, Pepe A, Dauchez M, Bochicchio B, Tamburro AM, Alix AJ. Structure and modeling studies of the carboxy-terminus region of human tropoelastin. Matrix Biol 2005;24:271–282. [PubMed: 15961300]

Franzblau, C.; Baris, B.; Lent, RW.; Salcedo, LL.; Smith, B.; Jaffe, R.; Crombie, G. Chemistry and biosynthesis of crosslinks in elastin. In: Balazs, EA., editor. Chemistry and Molecular Biology of the Intracellular Matrix. 1. Academic Press; New York: 1969. p. 617-639.

Franzblau C, Pratt CA, Faris B, Colannino NM, Offner GD, Mogayzel PJJ, Troxler RF. Role of tropoelastin fragmentation in elastogenesis in rat smooth muscle cells. J. Biol. Chem 1989;264:15115–15119. [PubMed: 2768256]

Gerber GE, Anwar RA. Structural studies on cross-linked regions of elastin. J. Biol. Chem 1974;249:5200–5207. [PubMed: 4854426]

Getie M, Schmelzer CE, Neubert RH. Characterization of peptides resulting from digestion of human skin elastin with elastase. Proteins 2005;61:649–657. [PubMed: 16161116]

Hance KA, Tataria M, Ziporin SJ, Lee JK, Thompson RW. Monocyte chemotactic activity in human abdominal aortic aneurysms: role of elastin degradation peptides and the 67-kD cell surface elastin receptor. J Vasc Surg 2002;35:254–261. [PubMed: 11854722]

Houghton AM, Quintero PA, Perkins DL, Kobayashi DK, Kelley DG, Marconcini LA, Mecham RP, Senior RM, Shapiro SD. Elastin fragments drive disease progression in a murine model of emphysema. J. Clin. Invest 2006;116:753–759. [PubMed: 16470245]

Hsiao H, Stone PJ, Toselli P, Rosenbloom J, Franzblau C, Schreiber BM. The role of the carboxy terminus of tropoelastin in its assembly into the elastic fiber. Connect. Tiss. Res 1999;40:83–95.

Kozel BA, Ciliberto CH, Mecham RP. Deposition of tropoelastin into the extracellular matrix requires a competent elastic fiber scaffold but not live cells. Matrix Biol 2004;23:23–34. [PubMed: 15172035]

Kozel BA, Rongish BJ, Czirok A, Zach J, Little CD, Davis EC, Knutsen RH, Wagenseil JE, Levy MA, Mecham RP. Elastic fiber formation: A dynamic view of extracellular matrix assembly using timer reporters. J. Cell. Physiol 2006;207:87–96. [PubMed: 16261592]

Kozel BA, Wachi H, Davis EC, Mecham RP. Domains in tropoelastin that mediate elastin deposition in vitro and in vivo. J. Biol. Chem 2003;278:18491–18498. [PubMed: 12626514]

Lansing AI, Roberts E, Ramasarma GB, Rosenthal TB, Alex M. Changes with age in amino acid composition of arterial elastin. Proc. Soc. Exp. Biol. Med 1951;76:714–717. [PubMed: 14844321]

Li DY, Toland AE, Boak BB, Atkinson DL, Ensing GJ, Morris CA, Keating MR. Elastin point mutations cause an obstructive vascular disease, supravalvular aortic stenosis. Hum. Molec. Gen 1997;6:1021–1028. [PubMed: 9215670]

Lowry OH, Gilligan DR, Katersky EM. The determination of collagen and elastin in tissues, with results obained in various normal tissues from different species. J. Biol. Chem 1941;139:795–804.

Mithieux SM, Weiss AS. Elastin. Adv. Protein Chem 2005;70:437–461.

Mithieux SM, Wise SG, Raftery MJ, Starcher B, Weiss AS. A model two-component system for studying the architecture of elastin assembly in vitro. J. Struct. Biol 2005;149:282–289. [PubMed: 15721582]

Partridge SM. Elastin. Adv. Prot. Chem 1962;17:227–302.

Price LS, Roos PJ, Shively VP, Sandberg LB. Valyl-alanyl-prolylglycine (VAPG) serves as a quantitative marker for human elastins. Matrix 1993;13:307–311. [PubMed: 8412988]

Rodgers UR, Weiss AS. Integrin alpha v beta 3 binds a unique non-RGD site near the C-terminus of human tropoelastin. Biochimie 2004;86:173–178. [PubMed: 15134831]

Rosenbloom J,G,W, Abrams W, Ornsten GN, Indik Z, Kucich U. Newly determined carboxy terminal sequences in tropoelastin: immunologic identification in insoluble elastin. Coll. Relat. Res 1986;6:423–433. [PubMed: 3545661]

Sato F, Wachi H, Ishida M, Nonaka R, Onoue S, Urban Z, Starcher BC, Seyama Y. Distinct steps of cross-linking, self-association, and maturation of tropoelastin are necessary for elastic fiber formation. J. Mol. Biol 2007;369:841–851. [PubMed: 17459412]

Schmelzer CE, Getie M, Neubert RH. Mass spectrometric characterization of human skin elastin peptides produced by proteolytic digestion with pepsin and thermitase. J. Chromatogr 2005;A 1083:120–126.

Shapiro SD, Endicott SK, Province MA, Pierce JA, Campbell EJ. Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. J. Clin. Invest 1991;87:1828–1834. [PubMed: 2022748]

Soskel NT, Sandburg LB. A comparison of six methods of extracting elastin residue from hamster lungs. Exp. Lung Res. Experimental lung research 1983;4:109–119.

Starcher BC, Galione MJ. Purification and comparison of elastins from different animal species. Anal. Biochem 1976;74:441–447. [PubMed: 822746]

Steinbuch M, Audran R. The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch. Biochem. Biophys 1969;134:279–284. [PubMed: 4982185]

Szabo Z, Crepeau MW, Mitchell AL, Stephan MJ, Puntel RA, Yin Loke K, Kirk RC, Urban Z. Aortic aneurysmal disease and cutis laxa caused by defects in the elastin gene. J. Med. Genet 2006;43:255–258. [PubMed: 16085695]

Urban Z, Michels VV, Thibodeau SN, Davis EC, Bonnefont J-P, Munnich A, Eyskens B, Gewillig M, Devriendt K, Boyd CD. Isolated supravalvular aortic stenosis: functional haploinsufficiency of the elastin gene as a result of nonsense-mediated decay. Hum. Genet 2000;106:577–588. [PubMed: 10942104]

Wise SG, Mithieux SM, Raftery MJ, Weiss AS. Specificity in the coacervation of tropoelastin: solvent exposed lysines. J. Struct. Biol 2005;149:273–281. [PubMed: 15721581]

Wrenn DS, Griffin GL, Senior RM, Mecham RP. Characterization of biologically active domains on elastin: Identification of a monoclonal antibody to a cell recognition site. Biochemistry 1986;25:5172–5176. [PubMed: 2429696]

Zhang MC, He L, Giro M, Yong SL, Tiller GE, Davidson JM. Cutis laxa arising from frameshift mutations in exon 30 of the elastin gene (ELN). J. Biol. Chem 1999;274:981–986. [PubMed: 9873040]

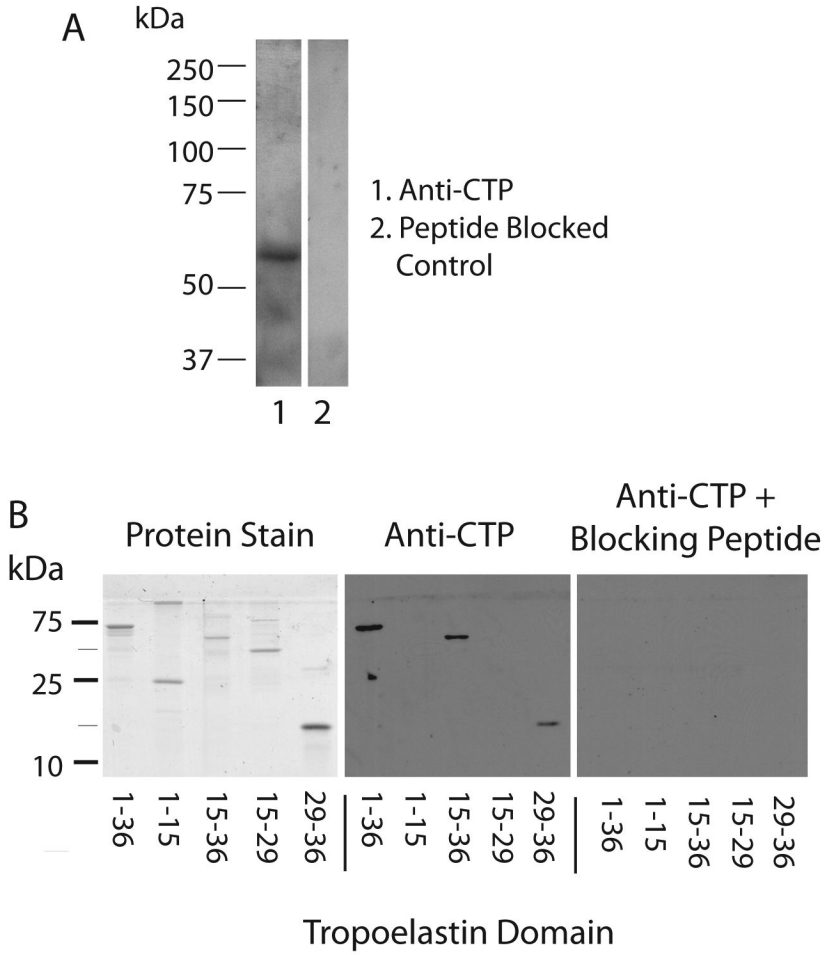


Figure 1. The CTP antibody specifically reacts with the C-terminus of tropoelastin in western blots

A) Western blot analysis of tropoelastin produced by FBAF cells using an antibody generated to the C-terminal 19 amino acids of bovine tropoelastin (CTP antibody). Lanes 1 and 2 contain equivalent amounts of a detergent extract of confluent FBAF cells. The transferred proteins in lane 1 were probed with the CTP antibody. Lane 2 was probed with the CTP antibody preincubated with 10μM CT-25 peptide. Blots 1 and 2 were developed/exposed simultaneously. **B)** Western blot analysis of bacterially expressed tropoelastin and tropoelastin fragments denoted by domain numbers. The left panel is a coomassie blue stained transfer to visualize protein loading. The center panel was probed with the CTP antibody. The right panel was probed with the CTP antibody that was pre-incubated with 10μM CT-25 peptide. The center and right panels were developed/exposed simultaneously.

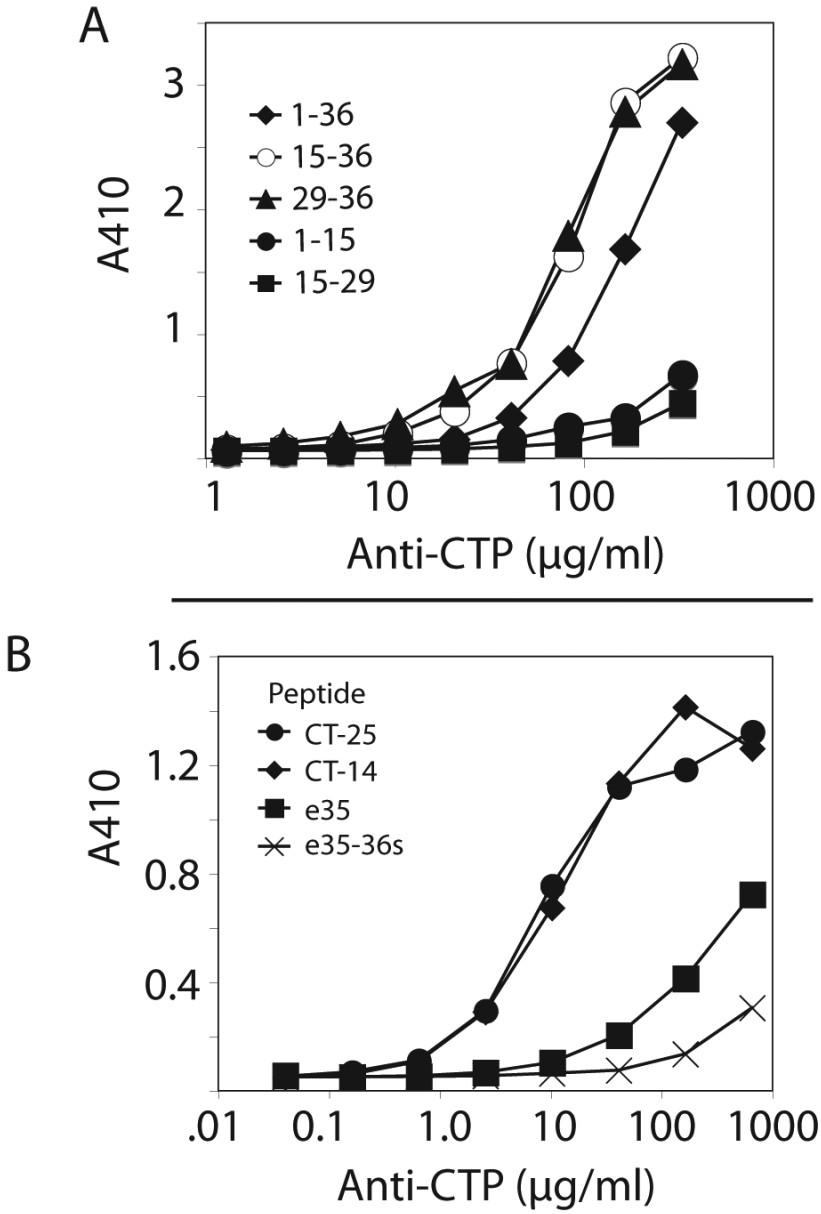


Figure 2. Reactivity of the CTP antibody for the domain-36 sequence is confirmed by ELISA assay
A) Dilution curves for the CTP antibody reacting with microtiter wells coated with full-length tropoelastin (domains 1-36, diamonds) or with fragments consisting of domains 15-36 (open circles), 29-36 (triangles), 1-15 (closed circles), and 15-29 (squares). **B)** Dilution curves for the CTP antibody reacting with microtiter wells coated with the CT-25 synthetic peptide (circles), CT-14 (diamonds), e35 (squares), or the scrambled peptide e35-36s (x's).

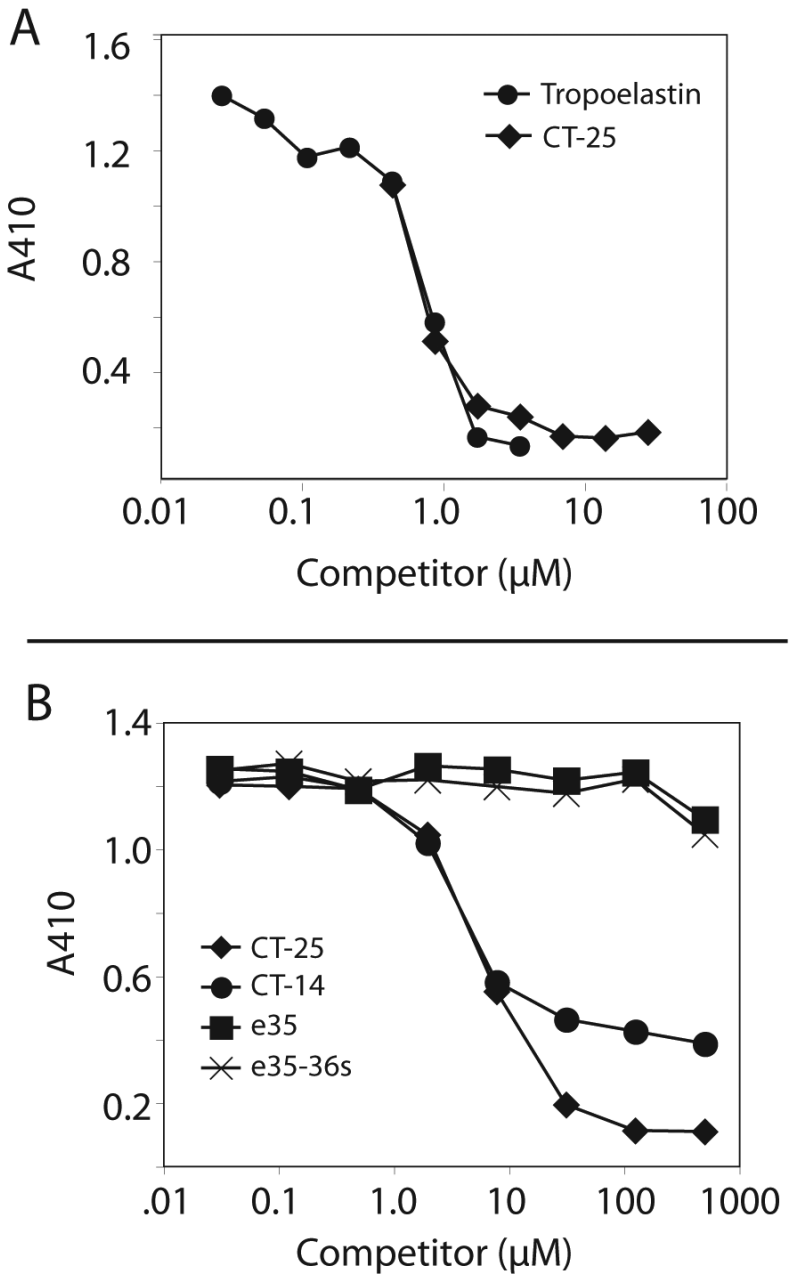


Figure 3. Fine mapping of anti-CTP epitope by competition assay

A) Competition assay showing that soluble tropoelastin (circles) and CT-25 peptide (diamonds) inhibit binding of the CTP antibody to a substrate coated with tropoelastin. **B)** Competition assay comparing the ability of CT-25 peptide (diamonds), CT-14 peptide (circles), e35 peptide (squares) or the scrambled e35-36s peptide (X's) to block binding of CTP antibody to a substrate coated with the CT-25 peptide.

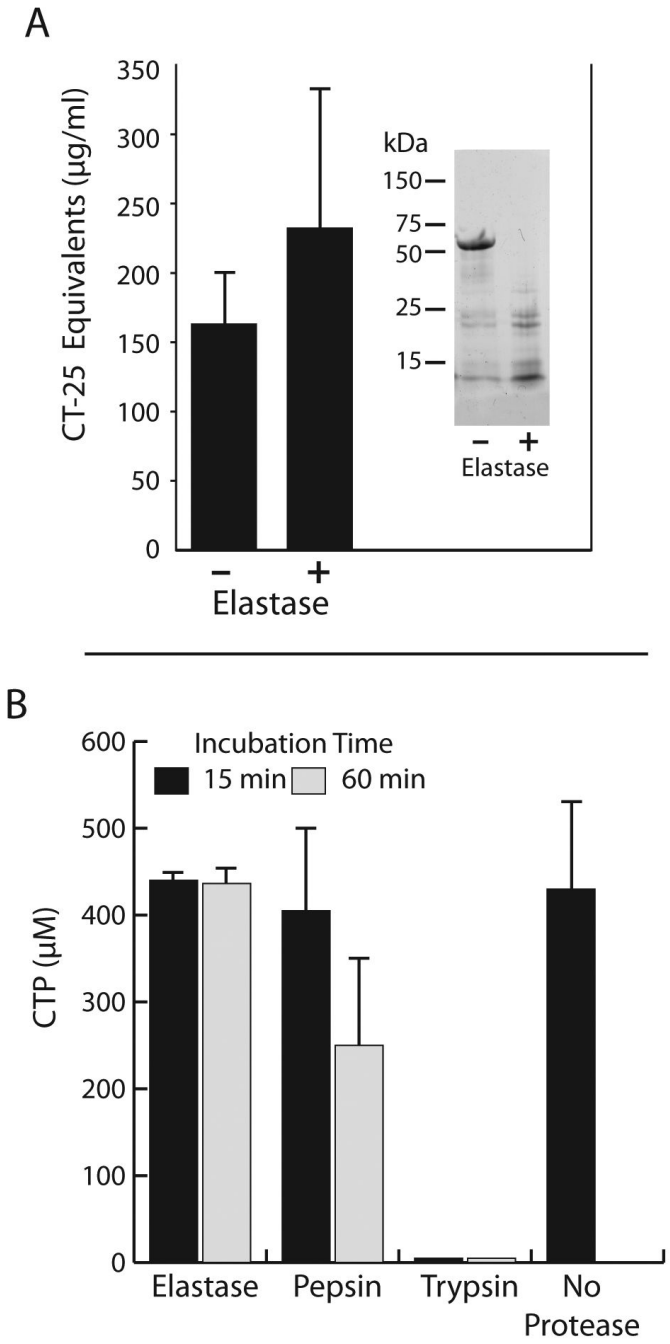
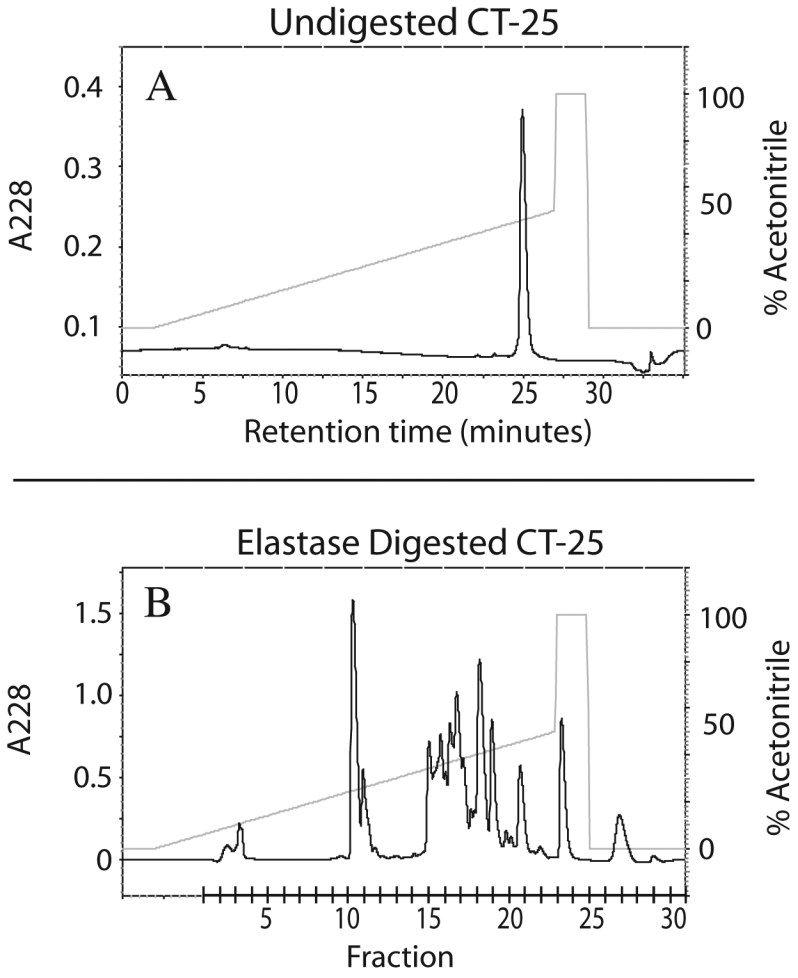


Figure 4. The epitope for the anti-CTP antibody in tropoelastin and in the CT-25 peptide is insensitive to elastase treatment

A) Full-length tropoelastin (200 µg/ml) was incubated for 1 hour at room temperature with or without 2µg/ml porcine pancreatic elastase. The digestion was stopped by the addition of PMSF to 1mM. Immunoreactive carboxy-terminal peptide in each sample was then determined by competitive immunoassay using the CT-25 peptide as a reference standard. The amount of measurable carboxy-terminal epitope expressed as CT-25 equivalents in undigested control tropoelastin (-elastase) was equivalent to that in elastase-digested tropoelastin (+elastase). The inset panel is a SDS-PAGE gel showing that tropoelastin incubated with elastase was fully digested. **B)** The epitope in the CT-25 peptide is insensitive to elastase and pepsin but destroyed

by trypsin. A 500μM solution of CT-25 peptide was digested with 1:100 (w:w) elastase, pepsin, or trypsin for either 15 or 60 minutes. CT-25 incubated without protease served as a control. The control, elastase and trypsin digestions were stopped by the addition of PMSF to 1mM. The pepsin digestion was stopped by neutralization with 1/10 volume of 1M TRIS pH=8.8. The samples were assayed by competitive immunoassay using the CT-25 peptide as a standard. Shown are mean ± standard deviation, n=4. The difference between 60 min pepsin digestion and no protease control was not significant ($p>.05$).



HPLC Fraction	Mass	Sequence
Undigested	2408	FGGALGALGFPGGACLGKSCGRKRK
Fraction 10	1235	CLGKSCGRKRK
Fraction 15	1722	GFPGGACLGKSCGRKRK
Fraction 16	591	FGGALGA
	614	GGALGALG
Fraction 17	1906	ALGFPGGACLGKSCGRKRK
	1835	LGFPGGACLGKSCGRKRK
Fraction 18	618	LGFPGGACLGKSCGRKRK
Fraction 23	1191	FGGALGALGFPGGA

Figure 5. The CT-25 peptide is cleaved at multiple sites by pancreatic elastase

CT-25 peptide was digested with pancreatic elastase using a 1:100 (w:w) enzyme to substrate ratio. The reaction was stopped after 1 hour with the addition of PMSF. Fragments were purified using reverse phase HPLC chromatography on a 4.6 × 25 cm C18 column using a 0-50% acetonitrile gradient. **Panel A** is the HPLC tracing of CT-25 peptide before digestion. **Panel B** is the HPLC tracing of CT-25 following elastase digestion. Fractions were dried and analyzed by mass spectrometry, giving the sequences shown at the bottom of the figure. The elution time scale for both chromatograms is the same. The grey line represents the acetonitrile gradient.

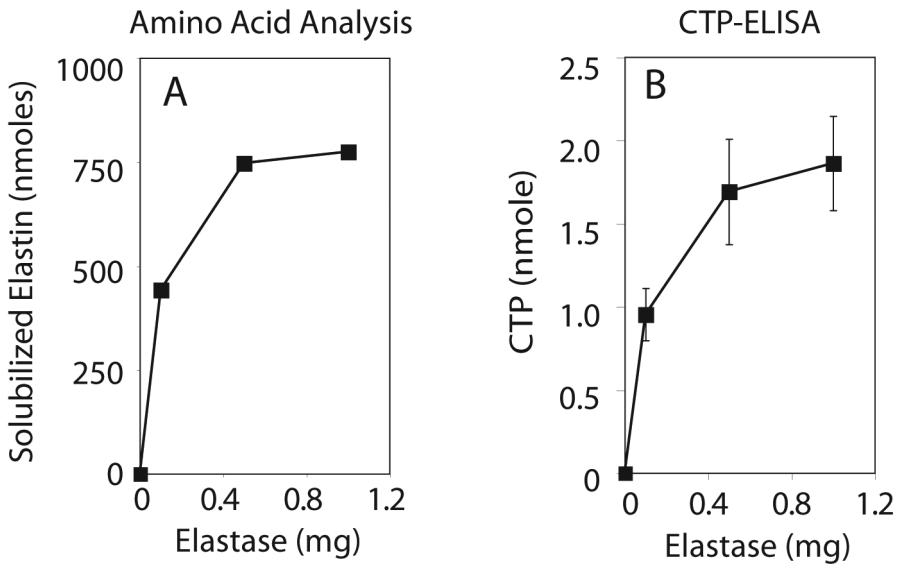


Figure 6. Elastase solubilization of the C-terminal fragment from mature elastin

Starcher purified elastin (50 mg) was digested with various amounts of porcine pancreatic elastase in 10mM HEPES, 10mM $CaCl_2$, pH=7.4. At 1 hour the reactions were stopped with the addition of PMSF. The digests were centrifuged for 2 minutes in a microfuge and the supernatant assayed for total soluble elastin using amino acid analysis **(left panel)** or competitive ELISA with the CTP antibody **(right panel)**. The amounts of soluble elastin and CTP released into the supernatant are given as nmoles. ELISA values are mean ± standard deviation, n=4.

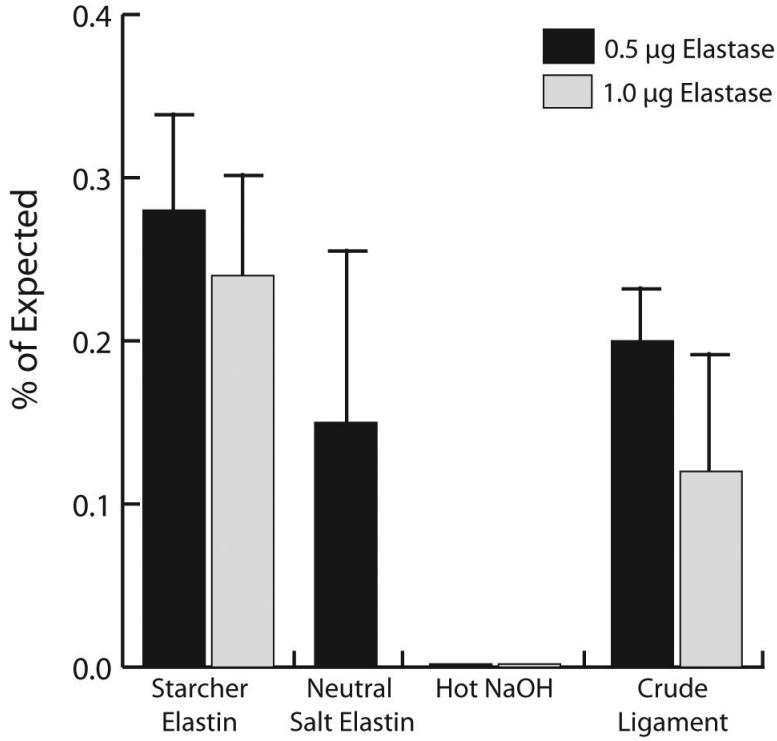


Figure 7. Relative content of immunoreactive C-terminus in elastin purified using different protocols

Fifty milligrams of insoluble elastin purified from bovine ligamentum nuchae using the Starcher, neutral salt, and hot NaOH methods was digested with 0.5 or 1.0 mg pancreatic elastase for one hour at 37°C. Crude, defatted ligamentum nuchae, digested using the same conditions, served as an unprocessed control. Following digestion, total solubilized elastin was quantified using desmosine and isodesmosine content (determined by amino acid analysis). Competitive ELISA quantified the amount of soluble fragment with an immunoreactive C-terminus. The amount of CTP is expressed as a percent of the expected based on the total amount of elastin solubilized. Shown are mean ± standard deviation, n=4.

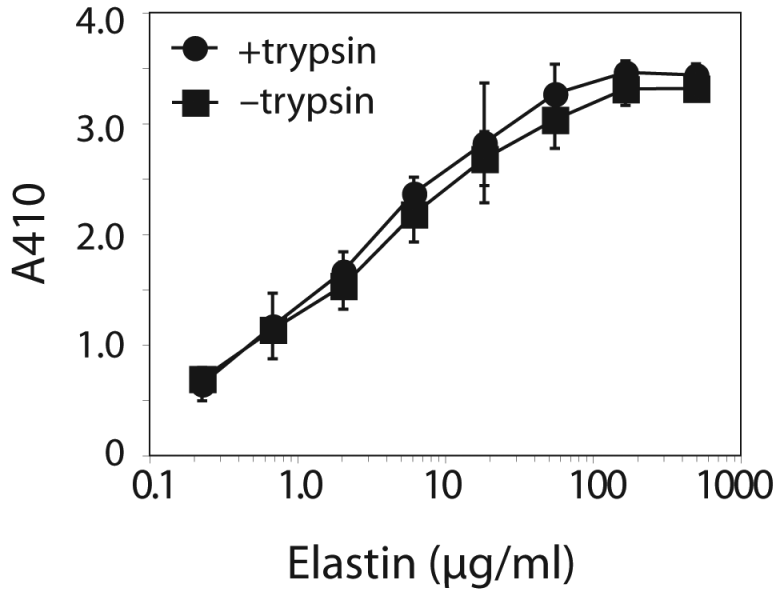


Figure 8. The epitope for the anti-CTP antibody in elastase-derived fragments from mature elastin is insensitive to trypsin digestion

Binding of CTP antibody to microtiter wells coated with increasing amounts of elastase-derived, partially purified anti-CTP reactive fragments from neutral salt purified elastin incubated with or without trypsin. Incubation of the fragments with trypsin prior to coating (circles) had no effect on antigenicity when compared to fragments incubated without trypsin (squares). Shown are mean ± standard deviation, n=4.

Table 1

Synthetic peptides used for epitope mapping of the CTP antibody. Sequences of domains 35 and 36 are provided for reference.

Name	Sequence
Exon 36	GGACLGKSCGRKRK
Exon 35	GKPPKPFGGALGALGFP
CTP	ALGFPGGACLGKSCGRKRK
CT-25	FGGALGALGFPGGACLGKSCGRKRK
CT-14	GGACLGKSCGRKRK
e35	FGGALGALGFP
e35-36s	GPKAFGKACSPARGGLCKGFPGRGLGKPGKG