Identification of Multiple Tropoelastins Secreted by Bovine Cells*

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High resolution gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis, cell-free translation, and elastin-specific antibodies were used to identify three tropoelastin isoforms secreted by bovine tissue and cells. Tropoelastin isolated from nuchal ligament and from conditioned culture medium or cell-matrix extracts of ligament fibroblasts and auricular chondrocytes resolved as three distinct bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with molecular weights of approximately 67,500 (tropoelastin I), 65,000 (tropoelastin II), and 62,000 (tropoelastin III). Three tropoelastin polypeptides with molecular mass 2–3 kDa higher than their corresponding tissue forms were also evident in cell-free translation products of ligamentum nuchae RNA, suggesting that each tropoelastin species is encoded by a unique mRNA. The presence of cysteine in all three tropoelastin isoforms was demonstrated by the incorporation of [35S]cysteine into newly synthesized tropoelastin polypeptides and by immunoreactivity with an antibody raised against a synthetic peptide that defines the cysteine-containing carboxyl-terminal region of tropoelastin. Immunological co-localization of the carboxy-terminal antibody with insoluble elastin in lung vasculature and parenchyma suggests that intact tropoelastin and not a processed form is incorporated into the elastin fiber.

Unlike the collagens, which have been characterized as a multigene family (Fleischmajer et al., 1985), elastin’s unique physical properties have precluded a thorough description of the complexities of the elastin phenotype. Despite accumulating evidence for multiple forms of tropoelastin, questions still remain as to their precise number, the structural relationships between them, and their cellular origin.

Elastin is a polymeric protein composed of individual tropoelastin molecules cross-linked one with another to form a functional elastomer. Most of what is known about elastin structure is based on biochemical and physicochemical characterization of uncross-linked tropoelastin. First isolated from elastic tissues of copper-deficient or lathyritic animals, the elastin precursor was identified as a single, nonglycosylated protein with a molecular weight of approximately 74,000.

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faint band slightly above but not completely separated from tropoelastin II. Best resolution was obtained when gels were used within 24 h of preparation.

The immunoblots in Fig. 2, lanes D and E, demonstrate that all three tropoelastins are reactive with polyclonal- and monoclonal-derived anti-elastin IgG.

**Tropoelastin from Cell Culture**—To determine whether the three tropoelastins extracted from ligament tissue are secretory forms of elastin, synthetic products of ligamentum nuchae fibroblasts and ear cartilage chondrocytes where radio-

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**TABLE I**

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<th>Amino acid composition (residues/1000) of purified ligament nuchae tropoelastin and insoluble elastin</th>
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b Low hydroxyproline values reflect the absence of ascorbate during the incubation period.

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**Fig. 2. SDS-polyacrylamide gel electrophoresis of purified tropoelastin.** Bovine tropoelastin purified from ligamentum nuchae was analyzed by electrophoresis using a Laemmli SDS-polyacrylamide gel (10% cross-link) (lane B) or an ultrathin, 7-12.5% gradient gel (lane C). Both gels were stained with silver. Lanes D and E show immunoblots of different preparations of ligament tropoelastin developed with anti-elastin monoclonal IgG; lane F is with preimmune serum. Gels were run under reducing conditions. Molecular mass standards (lanes A and G) include myosin (205 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

**Fig. 3. Tropoelastins synthesized by cultured cells.** Fluorographs of [3H]leucine-labeled tropoelastin immunoprecipitated with a monoclonal antibody from culture medium of ligament fibroblasts (lane A) or auricular chondrocytes (lane B) and chromatographed on gradient SDS-PAGE gels as described in the legend to Fig. 2. Immunoprecipitates from the cell layer of cultured chondrocytes at 4 days (lane D) and 4 weeks (lane E) post-confluence. Lanes C and F are immunoprecipitates from medium and cell layer extracts, respectively, using normal ascites IgG. *C-Labeled molecular mass standards (lane G) include myosin (205 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (66 kDa), egg albumin (46 kDa), and lactoglobulin A (18.4 kDa).

labeled with [3H]leucine and tropoelastin was precipitated from culture medium with anti-elastin monoclonal IgG. Fig. 3 shows that all three tropoelastins were present in the medium of both cell types.

To evaluate whether multiple tropoelastins are also associated with the extracellular matrix of cultured cells, ear chondrocytes were cultured overnight with [3H]leucine in the presence of β-aminopropionitrile and soluble components in the cell layer were extracted with acetic acid. Chondrocytes were chosen for this experiment because they retain a large percentage of secreted tropoelastin in the extracellular matrix (Mecham et al., 1981; Starcher and Mecham, 1981). SDS-PAGE of immunoprecipitates showed that no tropoelastin was associated with the cell layer at 4 days post-confluence but after 4 weeks of culture all three tropoelastins were present in a ratio similar to that seen for the soluble proteins.
in culture medium (Fig. 3, lane E).

Quantification by scanning densitometry of numerous gel fluorographs showed that the ratio of the three tropoelastins secreted by cultured cells was variable, even when isolated from the same cell type. Tropoelastins II and III were the major elastin products with tropoelastin I appearing as a minor product in most instances. As an average, the relative proportion of tropoelastin I, II, and III secreted into culture medium by first passage cells was approximately 1:3:2, respectively (Fig. 4).

Tropoelastin in Cell-free Translation of Elastin RNA—To examine if the multiple tropoelastins are encoded by unique RNAs, proteins in reticulocyte lysate translations of ligament tissue RNA were analyzed by high resolution SDS-PAGE. Using guanidine thiocyanate extraction and cesium chloride density centrifugation, approximately 16.8 μg of RNA were isolated per gram (wet weight) of ligament tissue. Cell-free translation of ligament RNA gave an approximately 16-fold stimulation over background (no RNA) in [3H]leucine and [35S]cysteine incorporation into trichloroacetic acid-precipitable protein. Electrophoresis of translation products on high resolution SDS-PAGE showed three major proteins with apparent molecular weights of 71,000, 67,500, and 65,000 (Fig. 5). Specific precipitation with anti-elastin monoclonal IgG confirmed that the three bands were tropoelastin. The molecular weights of the cell-free translation products are 2000–3000 larger than their corresponding tissue forms (see Fig. 4).

Evidence for a Cysteine-containing Region Near the Carboxyl Terminus—The presence of cysteine in newly synthesized tropoelastin polypeptides is consistent with elastin gene structural analysis that predicts a unique, cysteine-containing amino acid sequence at the carboxyl-terminal end of the molecule (Cicila et al., 1985). The presence of such a sequence on tropoelastin was confirmed using a monospecific antiseraum generated to a synthetic peptide with an amino acid sequence determined from nucleotide sequencing of this region of the gene. As shown in Fig. 6, all three tropoelastin bands were immunoreactive with the carboxyl-terminal antibody. Interestingly, this antibody reacted only with intact tropoelastin and did not recognize lower molecular weight degradation products of tropoelastin that are otherwise immunoreactive with a monoclonal antibody specific for a repeating, internal hexameric sequence in elastin (lane B). Each lane contains an equivalent loading of tropoelastin.

**DISCUSSION**

With the identification of multiple tropoelastins, much interest has centered on identifying the exact nature of these proteins. Using severe denaturing conditions to extract and purify tropoelastin from chicken aorta, Rich and Foster (1984) showed that tropoelastin a had more polar amino acids, 5–8 times the number of cysteine residues, and a higher percentage of hydroxyproline than tropoelastin b. Differences in molecular weight, amino acid composition, isotope incorporation, antigenic determinants, and peptide maps imply significant differences between the two tropoelastins (Karr and Foster, 1983). Other studies, however, have identified tropoelastin forms that do not differ as extensively. In chick embryo artery cells, two distinct tropoelastin polypeptides were observed only when unhydroxylated proteins were extracted from cells incubated with α,α-bipyridine or 3,4-dehydroproline (Saunders and Grant, 1985). It was suggested in these studies that microheterogeneity induced by partial and random hydrox-
ylation of the proteins in normal cultures was sufficient to obscure differences in their electrophoretic mobilities. Peptide mapping of the underhydroxylated tropoelastins indicated close identity between the two molecules with minor differences best explained by the presence of a peptide sequence in tropoelastin a that is absent in tropoelastin b.

These studies with chicken tissues suggest that tropoelastin a and tropoelastin b represent distinct gene products. In rat aorta, however, multiple tropoelastins may result from proteolytic processing of a single secreted tropoelastin precursor. Chipman et al. (1985) identified a single 77,000-dalton tropoelastin-like molecule in cultures of rat aortic smooth muscle cells that appeared to be processed extracellularly to a 71,000-dalton form. Such a precursor-product relationship between the two proteins raises the possibility that, even in other systems, tropoelastin b is a processed form of tropoelastin a.

Using high resolution SDS-PAGE and antibodies specific for elastin we have identified three tropoelastin isoforms with molecular weights of 67,500, 65,000, and 62,000 in tissue extracts of ligamentum nuchae as well as in culture medium and cell-matrix extracts of ligament fibroblasts and chondrocytes from auricular cartilage. On standard Laemmli gels, the three isoforms co-migrated as a single broad band at $M_r = 66,000$. The ratio of tropoelastin I, II, and III (approximately 1:3:2, respectively) was similar in first passage cultures of each cell type but fluctuated unpredictably as the cells aged in culture or underwent repeated cell division.

Evidence that the tropoelastins represent unique secreted proteins is supported by the following observations. First, tropoelastin I, II, and III were identified as cell-free translation products of ligament tissue RNA. All three proteins were approximately 2–3 kDa larger than the tissue forms, which is consistent with the presence of a "signal" peptide on the amino terminus of each molecule, as has been established for chicken (Foster et al., 1981) and sheep (Davidson et al., 1982) tropoelastins. Thus, each protein appears to have been encoded by a unique RNA. Second, the absence of hydroxylation and glycosylation reactions in cell-free systems argues against these modifications as explanations for the observed differences in electrophoretic mobilities. Third, each of the three tropoelastins reacts with antiserum having specificity for a sequence at the carboxyl terminus of the molecule. If molecular weight differences between the three proteins were to arise by proteolytic removal of this carboxyl-terminal sequence, tropoelastin II and III would not react with the antibody.

Available physical and chemical information do not yet explain the observed variation in size or chromatographic behavior of the three proteins. Because the molecules have an intact amino and carboxyl terminus, differences between the proteins must be internal to these sites. When subjected to isoelectric focusing, all three tropoelastins migrated as a single band at the basic end of the pH gradient (not shown), confirming that structural differences between the isoforms do not include alterations which change the overall net charge of any one of the molecules. Furthermore, the co-migration of all three tropoelastins as a single peak on reverse phase HPLC suggests that the proteins have similar physical properties.

In immunoprecipitation and Western blot studies with monoclonal antibodies, it was observed that the characteristic degradation products of tropoelastin (Mecham and Foster,
1977) often migrate on SDS-PAGE as trimers with molecular weight differences identical to those observed between the parent proteins. This fortuitous form of peptide mapping supports the existence of common protease-susceptible sites and large segments of sequence homology between the three isoforms.

The interaction of all three tropoelastins with anticarboxyl-terminal antibody establishes that at least a portion of this novel sequence, predicted from nucleotide sequence analysis of the elastin gene, and extending from the carboxyl terminus to residue -17 (Cicila et al., 1985), is present on the molecule even though a corresponding sequence has not been found in tryptic peptides from tropoelastin (Sandberg et al., 1985). The distinctive features of this sequence include the presence of cysteine residues and a clustering of highly basic amino acids. While the basic amino acids may facilitate the interaction between tropoelastin and acidic components of elastic fiber microfibrils, the migration of tropoelastin as a trimer on SDS-PAGE under nonreducing conditions and the ease with which tropoelastin can be extracted from ligament tissue in the absence of reducing agents suggest that the 2 cysteine residues form an intramolecular bond or exist as free sulfhydryls. However, we cannot exclude the possibility that the formation of intermolecular disulfide bonds between tropoelastin monomers or between tropoelastin and other components of the extracellular matrix is a time-dependent process such that extractable tropoelastin represents a pool that has not yet undergone disulfide linkage.

Conclusions about the role of the carboxyl-terminal segment of tropoelastin must remain speculative but since the sequence remains associated with insoluble elastin, one can speculate that the carboxyl-terminal region might be important for some phase of fibril formation. It is interesting that only intact tropoelastin I, II, and III and not the characteristic lower molecular weight degradation fragments react with the carboxyl-terminal antibody (Fig. 6). These findings, together with the demonstration of intact tropoelastin associated with the extracellular matrix of cultured cells and tissues, suggest that only intact tropoelastin and not a form that is processed at the carboxyl terminus becomes incorporated into the elastin fiber.

It has yet to be established whether the three tropoelastin arise from the same or different genes. In situ hybridization experiments using a human cDNA clone and human metaphase chromosomes have identified only one elastin gene locus in the human genome localized to the q31–qter region of chromosome 2 (Emanuel et al., 1985). If the tropoelastin variants are encoded by a single elastin gene, then differences must arise from alternative splicing of the primary transcript. Preliminary S-1 mapping experiments have suggested the occurrence of alternative splicing events in elastin mRNA isolated from bovine ligamentum nuchae (Yoon et al., 1984). Clearly, further studies are required to establish the genetic origins of the three bovine tropoelastins.

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Supplementary Material to
Identification of Multiple Tropoelastin Species by Bone Cells

David S. Whalen, William C. Parker, Loran A. Whitehouse, Edmund C. Crouch

Experimental Procedures

Cell Culture. Fibroblasts from aortic muccosa were grown from explants of fetal bone tissue as described (Mecham et al., 1981). Chondrocytes from bovine ear cartilage were secured as described (Quintana et al., 1979). Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antiseptic, nonessential amino acids, and 10% fetal bovine serum.

Radiolabeling and Immunoprecipitation of Tropoelastin. Cell cultures were incubated overnight in labeling medium containing 100 nCi (4-6)H-leucine (Amersham, Arlington, IL) or 100 nCi 1(35)S-sulfate (ICN, Irvine, CA), and was shown to have the correct composition by amino acid analysis (Sandberg and Wilt, 1982). Supernatants were then collected, and the supernatant was dialyzed against buffer (ammonium sulfate, pH 7.0, 0.1 M NaCl). The precipitated samples were then washed for 0.5 h under vacuum at 4°C, and the precipitated material was then washed and lyophilized.

Production of Monoclonal Antibody to Carboxy Terminal Regions of Tropoelastin. The monoclonal antibody, which recognized a polypeptide of molecular weight 75,000 and a polypeptide of molecular weight 85,000, was obtained by injection of bovine tissues as described (Ward et al., 1979). Each reaction was incubated with 100 μg/ml of rabbit IgG and with 10 μg/ml of rabbit IgG conjugated to fluorescein isothiocyanate. The resulting sera were then absorbed with Sepharose (Pharmacia) to remove any anti-IgG or anti-BSA. The resulting sera were then used to prepare affinity-purified IgG for use in the immunoprecipitation procedure as described.

Immunoprecipitation of Purified Tropoelastin. Tropoelastin was purified from trypsin digests of bovine aorta using a fast protein liquid chromatography column. The purified tropoelastin was then dialyzed against 0.1 M NaCl and 0.01 M Tris-HCl, pH 7.4. The purified tropoelastin was then dialyzed against 0.1 M NaCl and 0.01 M Tris-HCl, pH 7.4. The purified tropoelastin was then dialyzed against 0.1 M NaCl and 0.01 M Tris-HCl, pH 7.4.

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