Fibroblast adhesion to recombinant tropoelastin expressed as a Protein A-fusion protein

Leonard E. GROSSO,* William C. PARKS,† Leeju WU †and Robert P. MECHAM‡§

Departments of *Pathology, †Dermatology, and ‡Respiratory and Critical Care Divisions, Department of Medicine, Jewish Hospital at Washington University Medical Center, and §Department of Cell Biology, Washington University Medical Center, St. Louis, MO 63110, U.S.A.

INTRODUCTION

Fetal bovine fibroblasts and auricular chondrocytes have a saturable, high-affinity, protease-sensitive elastin receptor (Hinek et al., 1988; Wrenn et al., 1988). This receptor mediates the chemotactic response of bovine fibroblasts and inflammatory cells to tropoelastin, and is necessary for the correct integration of secreted monomeric tropoelastin into the extracellular matrix (Senior et al., 1980, 1982, 1984; Hinek et al., 1988). Chemotaxis studies have identified the hydrophobic repeating hexapeptide, VGVAPG, of tropoelastin as a receptor-binding site (Senior et al., 1984; Wrenn et al., 1986; Mecham et al., 1989). Analysis of affinity-purified elastin receptor shows that it is a complex composed of three proteins with molecular masses of 67, 61 and 55 kDa (Hinek et al., 1988; Wrenn et al., 1988; Mecham et al., 1989). The 67 kDa protein, a peripheral membrane protein with lectin-like properties, binds tropoelastin (Hinek et al., 1988; Mecham et al., 1989).

Although previous experiments using purified bovine tropoelastin defined several properties of the bovine elastin receptor, detailed biochemical analysis of the interaction of tropoelastin with the elastin receptor has been hampered by problems in isolating sufficient quantities of tropoelastin for ligand–receptor binding studies. In addition, chemical modifications of tropoelastin, such as iodination, often result in a biologically inactive protein (Wrenn et al., 1988). Furthermore, bovine tropoelastin is synthesized as a heterogeneous mixture of protein isoforms that result from extensive alternative splicing of the primary RNA transcript (Wrenn et al., 1987; Parks et al., 1988a; Yeh et al., 1989). This multiplicity may complicate the interpretation of binding data if the individual isoforms have different affinities for the elastin receptor.

To circumvent the problems encountered with tropoelastin isolated from elastin-rich tissues, we utilized recombinant-DNA methodology to produce sufficient quantities of tropoelastin to study receptor–ligand interactions. We report here the construction of plasmid pSE76 containing a modified bovine tropoelastin gene fused to an inducible Protein A gene. *Escherichia coli* containing pSE76 produces an easily purified fusion protein. Cleavage of the fusion protein with CNBr released biologically active rTROPO.

MATERIALS AND METHODS

Reagents

Restriction enzymes, T4 DNA polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, RNAase H, EcoRI methylase, packagene and pGEM-4z were purchased from Promega (Madison, WI, U.S.A.). Mung bean nuclease, Ig–Sepharose, pRT12T and E. coli strain N4830-1 were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Avian-myeloblastosis-virus reverse transcriptase was from Seikagaku America (St. Petersburg, FL, U.S.A.). Sequenase, dideoxy-NTPs and dNTPs were from U.S. Biochemical Corp. (Cleveland, OH, U.S.A.). DIFCO Casamino acids were purchased from Fisher Scientific (St. Louis, MO, U.S.A.). Tryptsin/EDTA for cell culture was purchased from GIBCO/Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). Turkey egg-white trypsin inhibitor, chicken egg-white ovalbumin, ampicillin, phenylmethanesulphonyl fluoride, benzamidine, e-aminohexanoic acid, BSA, octanoic acid and pepstatin A were from Sigma (St. Louis, MO, U.S.A.). Vinyl e.i.s.a. plates were obtained from Costar (Cambridge, MA, U.S.A.). CNBr was from Kodak (Rochester, NY, U.S.A.). Iodo-Beads were from Pierce (Rockford, IL, U.S.A.). [γ-33P]ATP (3000 Ci/mmole) and [γ-32P]dCTP (3000 Ci/mmole) were from ICN (Leslie, IL, U.S.A.). [α-32P]dATP (1250 Ci/mmole) was purchased from Dupont (Wilmington, DE, U.S.A.). 125I was from Amersham (Arlington Heights, IL, U.S.A.).

Oligonucleotides

Oligonucleotides were synthesized by the cyanomethyl phosphoramidite method by using an Applied Biosystems DNA synthesizer model 380 A (Foster City, CA, U.S.A.) and purified by de-naturing polyacrylamide–gel electrophoresis. Double-stranded oligomers were prepared by annealing gel-purified complementary oligomers.

Abbreviations used: VGVAPG, the hexapeptide Val-Gly-Val-Ala-Pro-Gly; rTROPO, recombinant bovine tropoelastin; Protein A, Protein A encoded by plasmid pRT12T; PBS, phosphate-buffered saline (120 mM-NaCl, 2.7 mM-KCI/10 mM-sodium phosphate, pH 7.4); HDBSA, heat-denatured BSA (1 mg/ml in PBS); EBS, Earle’s balanced salt solution (116 mM-NaCl, 5.4 mM-KCl, 1.2 mM-NaH2PO4, 6 mM-Dextrose, 1 mM-MgSO4, 2 mM-CaCl2, 30 mM-Hepes, pH 7.4); TSTHE, 50 mM-Tris, 150 mM- NaCl, 0.05% Tween 20, 5 mM-EDTA, pH 7.6.

* To whom correspondence should be addressed.
RNA isolation and cDNA library construction

Total cellular RNA from the nuchal ligament of a 15-day calf was isolated by using guanidine isothiocyanate/CsCl (Wrenn et al., 1987), and poly(A)-containing RNA was obtained by two cycles of oligo(dT)-cellulose chromatography. Oligo(dT)-primed first-strand cDNA synthesis was achieved with avian-myeloblastosis-virus reverse transcriptase, and second-strand cDNA synthesis was performed as described by Gubler (1987). Duplex DNA was treated with mung-bean nuclease to remove hair-pin structures and T4 DNA polymerase was used to create blunt-ended DNA molecules. The duplex DNA was then methylated with EcoR1 methylase and ligated to EcoR1 linkers. After EcoR1 digestion, the cDNA was size-selected by ACA-34 column chromatography and spermine precipitation. The size-selected cDNA was ligated to phosphatase-treated EcoR1-digested λgt-11 arms, packaged and amplified in E. coli strain Y1088. Examination of the bovine ligament λgt-11 library showed 3.6 × 10^7 independent recombinant events with 90% of the bacteriophage containing cDNA inserts as assessed by β-galactosidase activity.

Tropoelastin cDNA isolation and characterization

Restriction-enzyme digests, DNA ligation reactions, agarose- and polyacrylamide-gel electrophoresis, gel purification of DNA fragments, and transformation of E. coli were by standard techniques (Sambrook et al., 1989). Oligonucleotides were radioactively labelled with T4 DNA polynucleotide kinase and [γ-32P]ATP under the manufacturer's recommended conditions. cDNA sequences were radioactively labelled by nick-translation in the presence of [α-32P]CTP (Meinkoth & Wahl, 1987). DNA sequencing used dyeoxy sequencing techniques as modified for plasmid DNA, Sequenase, [α-32P]dATP and sequencing primers corresponding to the SP6 and T7 promoters of pGEM-4z (Mierendorf & Pfeifer, 1987). Internal DNA sequence information was obtained from restriction fragments subcloned in pGEM-4z. Primers corresponding to sequences 5′ and 3′ of the multiple cloning site of pRIT2T were used to sequence the junctions of cDNA and plasmid (Nilsson et al., 1985a).

By screening the λgt-11 library with an oligonucleotide corresponding to the known DNA sequence encoding the C-terminal 7 amino acids and a cDNA (T66) corresponding to an internal region of tropoelastin (Yeh et al., 1987), bacteriophages containing large fragments of the coding sequence of bovine tropoelastin were selected. After plaque purification, bacteriophage DNA was isolated by the method of Helms et al. (1985). After preliminary characterization by restriction digest and agarose-gel electrophoresis, phage 11-4 was selected for further studies. Comparison with published sequences indicated that an EcoR1/Smal restriction fragment contained the coding sequence (Fig. 1a; Yeh et al., 1989). To characterize the coding region completely the EcoR1/Smal restriction fragment was subcloned; sequence analysis showed that it was composed of tropoelastin exons 12–13 and 15–36. The deletion of exon 14 is consistent with the alternative splicing of the region (Yeh et al., 1989). The Hind3/Smal fragment encoding contiguous exons 15–36, including the region coding for the VGAVPG repeat (exon 24), was used in the construction of the tropoelastin fusion gene (Fig. 1a).

Fusion-gene construction

To align the reading frames of the Protein A′ gene of prIT2T and the tropoelastin cDNA, an EcoR1/Hind3 oligonucleotide was ligated to the 5′ end of the Hind3/Smal tropoelastin fragment (Figs. 1a and 1b). After insertion into the EcoR1/Smal region of pGEM-4z, the EcoR1/SalI fragment (containing the Smal/SalI region of the polylinker of pGEM-4z at the 3′ end) was inserted into the EcoR1/SalI region of the multiple cloning site of prIT2T (Nilsson et al., 1985a,b). E. coli strain N4830-1 was transformed with this prIT2T-derived plasmid. Plasmid DNA was isolated from the transformants. The structure of pSE76 was verified by restriction digestion and sequencing of the prIT2T-cDNA junctions.

Fusion-protein characterization

Cultures (1 ml) of pSE76 containing E. coli strain N4830-1 were grown to stationary phase at 30 °C in LB medium containing 50 μg of ampicillin/ml. Expression of the fusion protein was induced by the addition of an equal volume of medium preheated to 68 °C. The incubation was continued at 42 °C. Samples were removed, and the bacteria were harvested by centrifugation. The bacterial pellet was resuspended in 5 vol. of SDS/PAGE sample buffer (Laemmli, 1970). The solution was sequentially sonicated and boiled to solubilize the bacterial proteins. Cell debris was removed by centrifugation, and the supernatant was recovered for analysis. SDS/PAGE and Western blotting were as previously described (Wrenn et al., 1987).

Expression of the fusion protein

Cultures of E. coli strain N4830-1 containing either pSE76 or prIT2T were grown in LB medium containing 50 μg of ampicillin/ml at 30 °C overnight. The culture was diluted with an equal volume of LB medium supplemented with 0.5% glucose and 1.5% Casamino acids. Bacterial growth was continued at 30 °C to an A660 of 1.5. An equal volume of LB medium containing glucose and Casamino acids preheated to 68 °C was
The culture pH was maintained between 7 and 7.4 by the periodic addition of 10 mM-NaOH. After 2 h at 42 °C, the *E. coli* were collected by centrifugation at 8000 g for 10 min at 4 °C. The cell pellet was washed with TSTE. After centrifugation, the bacterial pellet was frozen on solid CO₂ and stored at −70 °C.

**Preparation of the cell lysate and purification of the fusion protein**

At the time of protein isolation, the cell pellet was thawed in TSTE containing 0.5 mm-phenylmethanesulphonyl fluoride, 5 mM-benzamidine, 0.2 mM-ε-aminohexanoic acid and 0.25 µg of pepstatin A/ml at 4 °C. The *E. coli* were lysed by sonication (4 × 1 min with Ultrasonic Sonicator model W-220 at maximum power). Cell debris was removed by centrifugation at 15000 g for 20 min at 4 °C. The supernatant was diluted with an equal volume of TSTE containing protease inhibitors and applied to an Ig-Sepharose column (1 cm diameter × 5 cm) with a flow rate of 30 ml/h. After extensive washing with TSTE and 10 mM-ammonium acetate, pH 5 (60-100 ml each), the retained protein was eluted with 0.5 M-ε-aminohexanoic acid, pH 3.5. Protein-containing fractions were pooled and freeze-dried. The fusion protein was treated with 70% (v/v) formic acid containing 20-30 mg of CNBr/ml at 30 °C for 5 h and the cleavage reaction was terminated by the addition of water. Residual CNBr and formic acid were removed by repeated freeze-drying. Protein concentrations were determined by the Bio-Rad (Richmond, CA, U.S.A.) protein assay with BSA as the standard. Tropoelastin and the cDNA encoding exons 15-36, including the known receptor-binding site of tropoelastin, were ligated into the truncated Protein A gene of pRIT2T (Nilsson et al., 1985a,b). To align the reading frames of the Protein A gene and the cDNA, an oligonucleotide linker was inserted. The resulting plasmid, pSE76, contained a fusion gene with a long open reading frame of 2246 bp. The open reading frame begins at the translation-initiation codon of the Protein A′ gene (encoded by pRIT2T) and extends to the C-terminal stop codon of the tropoelastin cDNA.

In *E. coli* N4830-1, transcription of the Protein A′ gene of pRIT2T is temperature-dependent. At 30 °C the locus is inactive, but at 42 °C transcription and subsequent translation occur. Since Protein A′ binds immunoglobulin with a high affinity, Western-blot analysis was used to detect proteins containing Protein A′ (Dahlman et al., 1989). Of importance, prior incubation of Western blots with non-specific human immunoglobulin blocks the Protein A′ interaction and allows detection of proteins containing specific determinants (Dahlman et al., 1989). At 30 °C, before induction, neither pRIT2T- nor pSE76-containing *E. coli* produced a protein detected by either non-specific rabbit immunoglobulin or anti-tropoelastin antibodies (Figs. 2a and 2b). After induction, *E. coli* containing pSE76, but not pRIT2T, produced a 71 kDa protein on silver-stained gels (Fig. 2c), which was detected on Western blots with non-specific immunoglobulin (Fig. 2a). Detection by BA4, a mouse monoclonal antibody reacting with the VGVAPG sequence of tropoelastin, verified that the tropoelastin component was present (Fig. 2b). The molecular mass agreed with the protein size predicted from the DNA sequence (Protein A′, 30 kDa; rTROPO, 40 kDa). In addition to the major 71 kDa band, several proteins of lesser molecular mass were present. These were considered to be the result of proteolytic degradation. Since the intensity of the 71 kDa band increased over 2 h without a relative increase in degradation products, rTROPO was isolated from *E. coli* 2 h after induction of protein synthesis (Figs. 2a and 2b).

In addition to potentially enhancing protein stability, inclusion of the Protein A′ gene simplified isolation of the fusion protein. The N-terminal region of Protein A′ is sufficient for high-affinity binding to immunoglobulin, and thus chromatography on Ig-Sepharose resulted in isolation of the 71 kDa fusion protein (Fig. 3a; Nilsson et al., 1985a; Dahlman et al., 1989). In addition to the predominant 71 kDa protein band, multiple bands of lesser molecular mass were seen; these were considered to represent degradation products of the tropoelastin component of the fusion protein. The short DNA fragment inserted between the Protein A′ gene and the tropoelastin cDNA incorporated a methionine residue near the N-terminus of the tropoelastin sequence (Fig. 1b). Since there is no methionine within the

**RESULTS AND DISCUSSION**

Although post-translational modifications of bovine tropoelastin, such as cleavage of the N-terminal signal peptide and hydroxylation of approx. 10% of its proline residues, take place, no glycosylation of bovine tropoelastin occurs (Hinek et al., 1988; Prosser & Mecham, 1988). Tropoelastin should therefore be an ideal protein to be produced in *E. coli*. Because the stability of eukaryotic proteins is often increased when they are expressed as fusion proteins (Marston, 1986, 1987), we inserted a 1420 bp cDNA encoding exons 15-36, including the known receptor-binding site of tropoelastin, into the truncated Protein A gene of pRIT2T (Nilsson et al., 1985a,b). To align the reading frames of the Protein A gene and the cDNA, an oligonucleotide linker was inserted. The resulting plasmid, pSE76, contained a fusion gene with a long open reading frame of 2246 bp. The open reading frame begins at the translation-initiation codon of the Protein A′ gene (encoded by pRIT2T) and extends to the C-terminal stop codon of the tropoelastin cDNA.

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tropoelastin sequence, this residue provides a convenient site to separate the Protein A chain from rTROPO (Smith, 1988). Western blots and silver-stained gels after cleavage of the isolated fusion protein with CNBr demonstrated a 40 kDa protein corresponding to rTROPO (Figs. 3a and 3b). Also encoded in the short oligonucleotide were two tyrosine residues C-terminal to the methionine. Iodination of bovine tropoelastin has previously been difficult, owing to a paucity of tyrosine residues (Wrenn et al., 1988). By incorporating tyrosine residues within the joining oligonucleotide, the recombinant protein, unlike native bovine tropoelastin, is easily iodinated (Fig. 3c). The quantity of rTROPO isolated, as determined by e.i.i.s.a., ranged from 2 to 10 mg per litre of E. coli culture.

Although these structural modifications were expected to be minor, it was important to confirm that rTROPO had appropriate biological properties. We used a fetal-bovine nuchal-ligament fibroblast adhesion assay to assess the activity of rTROPO. Late-gestation nuchal-ligament fibroblasts adhered strongly to surfaces coated with rTROPO (Table 1). The adherent cells maintained their spherical morphology and did not spread as did those plated on fetal-calf-serum-coated wells (results not shown). In agreement with previous studies, Protein A supported only minimal fibroblast adhesion (Table 1; Maeda et al., 1989). The attachment of cells to rTROPO and not to Protein A indicated that tropoelastin sequences and not Protein A fragments or other contaminants isolated from the bacterial culture mediated fibroblast adhesion.

Further documentation of the specificity of adhesion was obtained in experiments using BA4 antibody (Wrenn et al., 1986). This antibody recognizes a determinant on tropoelastin

![Fig. 2. Expression of Protein A-tropoelastin fusion protein by E. coli](image)

Total protein was prepared from pR1T2T- and pSE76-containing E. coli and analysed by SDS/PAGE with Western-blot analysis. (a) and (b) are Western-blot analyses of 7.5–12.5% polyacrylamide gradient gels; lanes 1 and 8 are from E. coli containing pR1T2T before and 100 min after shifting the culture temperature to 42 °C. Lane 2 is from E. coli containing pSE76 before induction. Lanes 3–7 are from pSE76-containing E. coli 20, 40, 60, 80 and 100 min, respectively, after shifting the culture temperature to 42 °C. (a) Western blot reacted with non-specific rabbit immunoglobulin. (b) Western blot reacted with BA4 after blocking with human immunoglobulin. (c) Silver-stained 7.5–12.5% polyacrylamide gradient gel. Lanes 1 and 2 are from pR1T2T-containing E. coli and lanes 3 and 4 are from pSE76-containing E. coli. Lanes 1 and 3 were before induction of the Protein A' locus. Lanes 2 and 4 were from E. coli 90 min after shifting the culture temperature to 42 °C. The arrow marks the position of the 71 kDa Protein A'-tropoelastin fusion protein.

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**Table 1. Nuchal-ligament fibroblast adhesion to rTROPO**

Late-gestation bovine nuchal-ligament fibroblasts (estimated gestational age 270 days) were plated on to wells precoated with rTROPO or Protein A. After 2.5 h at 37 °C, the non-adherent cells were washed away and the adherent cells quantified.

<table>
<thead>
<tr>
<th>Protein concn. (µg/ml)</th>
<th>Cell adhesion (A410)</th>
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<tbody>
<tr>
<td></td>
<td>rTROPO</td>
</tr>
<tr>
<td>5</td>
<td>0.11±0.03</td>
</tr>
<tr>
<td>10</td>
<td>0.99±0.13</td>
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<tr>
<td>20</td>
<td>1.50±0.05</td>
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**Table 2. Inhibition of fibroblast adhesion to rTROPO by monoclonal antibodies to the bovine elastin-receptor-binding site of tropoelastin**

Late-gestation bovine nuchal-ligament fibroblasts (estimated gestational age 270 days) were plated on to wells precoated with rTROPO (20 µg/ml) or fetal-calf serum (FCS) diluted 1:50 in PBS followed by incubation with either BA4 or immunoglobulin isolated from control ascites antibody diluted in HDBSA. After 2 h at 37 °C, the non-adherent cells were washed away and the adherent cells quantified.

<table>
<thead>
<tr>
<th>Antibody concn. (µg/ml)</th>
<th>Cell adhesion (A410)</th>
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<tr>
<td></td>
<td>rTROPO</td>
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<td></td>
<td>BA4</td>
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<tr>
<td>0.5</td>
<td>1.42±0.03</td>
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<td>0.5</td>
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<tr>
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<tr>
<td>50</td>
<td>0.07±0.02</td>
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Expression of tropoelastin by *Escherichia coli*

Fig. 3. Isolation and characterization of rTROPO

(a) Silver-stained SDS/polyacrylamide (7.5-12.5% gradient) gel. Lane 1, *E. coli* protein eluted from the Ig-Sepharose column. Lane 2, protein eluted from Ig-Sepharose cleaved with CNBr (rTROPO). (b) Western-blot analysis of rTROPO. Lane 1 (7.5-12.5% polyacrylamide gradient gel) reacted with a rabbit polyclonal anti-tropoelastin antibody; lane 2 (12.5% polyacrylamide gel) reacted with BA4. (c) Autoradiogram of iodinated rTROPO (7.5-12.5% polyacrylamide gradient gel). The arrows indicate the position of the 40 kDa rTROPO.

previously identified to participate in binding to the 67 kDa elastin receptor (Senior et al., 1984; Mecham et al., 1989). Although BA4 inhibited fibroblast adhesion to rTROPO (Table 2), BA4 had no affect on adhesion to fetal-calf serum (Table 2). Immunoglobulin isolated from control ascitic fluid had no affect on rTROPO-dependent adhesion (Table 2). These findings confirm the specificity of the interaction and strongly suggest that the 67 kDa bovine elastin receptor is involved in fibroblast adhesion.

Since expression of the bovine elastin receptor on ligament cells is developmentally regulated, we investigated the adhesion of early-gestation pre-elastogenic nuchal-ligament fibroblasts to rTROPO (Mecham et al., 1981, 1984a). Unlike late-gestation fibroblasts, these fibroblasts do not synthesize tropoelastin or demonstrate chemotaxis to tropoelastin (Mecham et al., 1981, 1984b; Parks et al., 1988a,b). While both early-gestation estimated gestational age 90 days (FCL90) and late-gestation fibroblasts (estimated gestational age 270 days (FCL270)) were equally adherent to fetal-calf-serum-coated wells, binding to rTROPO (200 μg/ml) was decreased by approx. 50% for the pre-elastogenic cells as compared with late-gestation fibroblasts (A410 for FCL90 = 0.74±0.16; A410 for FCL270 = 1.66±0.02). In addition to demonstrating the developmental regulation of adhesion of rTROPO, these findings also are consistent with the 67 kDa elastin receptor mediating nuchal-ligament fibroblast adhesion to rTROPO.

The ability to produce and isolate easily large quantities of a biologically active recombinant tropoelastin should facilitate studies of the tropoelastin–elastin–receptor interaction. With the minor structural modifications introduced, rTROPO is potentially a more useful ligand than native bovine tropoelastin. These features should allow assessment of the role of the elastin receptor in cell growth, development and elastogenesis.

Additional features of this expression system deserve mention. First, it is now possible to isolate a single tropoelastin isoform and study its physical and biological properties. In addition, the biological properties of specific regions of tropoelastin can now be investigated. The ability to introduce either specific amino acid changes via site-directed mutagenesis or to delete specific sequences of tropoelastin allows the study of unique proteins. Experiments with these recombinant proteins in conjunction with studies using chemically synthesized peptides may identify domains of tropoelastin with unrecognized biological functions. From a combination of these techniques and approaches, it is expected that information pertaining to the interactions of tropoelastin with both cells and matrix macromolecules may be gained.

Note added in proof (received 4 December 1990)

While this paper was under review, Indik et al. (1990) published a recombinant expression system for human tropoelastin.

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L. E. Grosso and others

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