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

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A bovine tropoelastin cDNA encoding exons 15–36 that includes the elastin-receptor binding site was expressed in *Escherichia coli* as a fusion protein with Protein A from *Staphylococcus aureus*. After isolation of the fusion protein by affinity chromatography on Ig–Sepharose, the tropoelastin domain was separated from plasmid-pR1T2T-encoded Protein A (Protein A′) by CNBr cleavage. Cell-adhesion assays demonstrated specific adhesion to the recombinant tropoelastin. Furthermore, the data indicate that interactions involving the bovine elastin receptor mediate nuchal-ligament fibroblast adhesion to the recombinant protein. In agreement with earlier studies of fibroblast chemotaxis to bovine tropoelastin, nuchal-ligament fibroblast adhesion demonstrated developmental regulation of the elastin receptor.

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.*, 1988*a*; 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, *Eco*R1 methylase, packagene and pGEM-4z were purchased from Promega (Madison, WI, U.S.A.). Mung bean nuclease, Ig–Sepharose, pR1T2T and *E. coli* strain N4830-1 were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Avian-myelobastosis-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.). Trypsin/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, ϵ-aminohexanoic acid, BSA, octanoic acid and pepstatin A were from Sigma (St. Louis, MO, U.S.A.). Vinyl e.l.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-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were from ICN (Leslie, IL, U.S.A.). [α-^{35}S]dATP (1250 Ci/mmol) was purchased from Dupont (Wilmington, DE, U.S.A.). ^{125}I was from Amersham (Arlington Heights, IL, U.S.A.).

Oligonucleotides

Oligonucleotides were synthesized by the cyanoethyl phosphoramitide method by using an Applied Biosystems DNA synthesizer model 380 A (Foster City, CA, U.S.A.) and purified by denaturing 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 pR1T2T; PBS, phosphate-buffered saline (120 mM-NaCl/2.7 mM-KCl/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 mMNaH_2PO_4, 6 mM-dextrose, 1 mM-$MgSO_4$, 2 mM-$CaCl_2$, 30 mM-Hepes, pH 7.4); TSTE, 50 mM-Tris, 150 mM-NaCl, 0.05% Tween 20, 5 mM-EDTA, pH 7.6.

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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 T_4 DNA polymerase was used to create blunt-ended DNA molecules. The duplex DNA was then methylated with *Eco*R1 methylase and ligated to *Eco*R1 linkers. After *Eco*R1 digestion, the cDNA was size-selected by ACA-34 column chromatography and spermine precipitation. The size-selected cDNA was ligated to phosphatase-treated *Eco*R1-digested λgt-11 arms, packaged and amplified in *E. coli* strain Y1088. Examination of the bovine ligament λgt-11 library showed 3.6×10^6 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-^{32}P]ATP under the manufacturer's recommended conditions. cDNA sequences were radioactively labelled by nick-translation in the presence of [α-^{32}P]dCTP (Meinkoth & Wahl, 1987). DNA sequencing used dideoxy sequencing techniques as modified for plasmid DNA, Sequenase, [α-^{35}S]dATP and sequencing primers corresponding to the SP6 and T7 promoters of pGEM-4z (Mierendorf & Pfeffer, 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 pR1T2T were used to sequence the junctions of cDNA and plasmid (Nilsson *et al.*, 1985*a*).

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 *Eco*R1/*Sma*1 restriction fragment contained the coding sequence (Fig. 1*a*; Yeh *et al.*, 1989). To characterize the coding region completely the *Eco*R1/*Sma*1 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 *Hin*d3/*Sma*1 fragment encoding contiguous exons 15–36, including the region coding for the VGVAPG repeat (exon 24), was used in the construction of the tropoelastin fusion gene (Fig. 1*a*).

Fusion-gene construction

To align the reading frames of the Protein A′ gene of pR1T2T and the tropoelastin cDNA, an *Eco*R1/*Hin*d3 oligonucleotide was ligated to the 5′ end of the *Hin*d3/*Sma*1 tropoelastin fragment (Figs. 1*a* and 1*b*). After insertion into the *Eco*R1/*Sma*1 region of pGEM-4z, the *Eco*R1/*Sal*1 fragment (containing the *Sma*1/*Sal*1 region of the polylinker of pGEM-4z at the 3′ end) was inserted into the *Eco*R1/*Sal*1 region of the multiple cloning site of pR1T2T (Nilsson *et al.*, 1985*a*,*b*). *E. coli* strain N4830-1 was transformed with this pR1T2T-derived plasmid. Plasmid DNA was isolated from the transformants. The structure of pSE76 was verified by restriction digestion and sequencing of the pR1T2T-cDNA junctions.

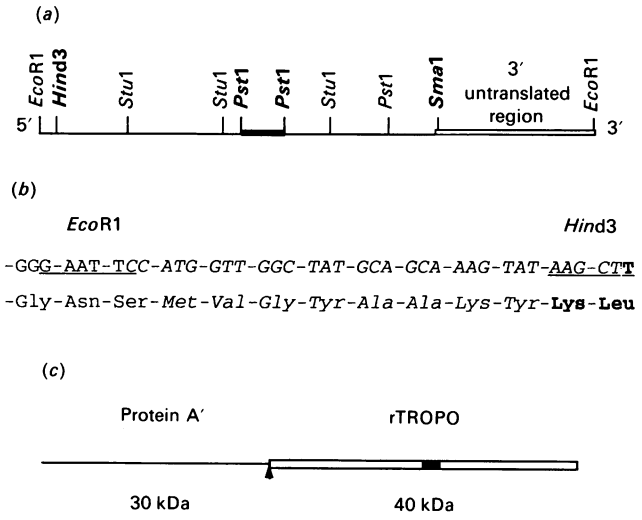

Fig. 1. DNA sequences used in the construction of rTROPO

(*a*) A partial restriction map of the λgt-11 tropoelastin cDNA clone. The *Pst*1 sites in bold flank the DNA sequence encoding the hydrophobic domain containing the VGVAPG (shown in black). The open box is the 3′ untranslated region of the clone. The *Hin*d3/*Sma*1 fragment was used to construct pSE76. (*b*) Sequence of the DNA oligonucleotide inserted between the Protein A′ gene of pR1T2T and the tropoelastin cDNA sequence. Above is the DNA and below is the encoded amino acid sequence. In roman type is the 3′ sequence of Protein A′, in italics is the linker sequence, and the 5′ tropoelastin sequence (from the *Hin*d3 site) is in bold. Restriction sites at the end of the linker are underlined. (*c*) Schematic drawing of the fusion protein containing rTROPO. The boxed region is rTROPO, with the VGVAPG domain in black. The arrowhead indicates the location of the oligonucleotide-encoded amino acids.

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 pR1T2T 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 A_{550} of 1.5. An equal volume of LB medium containing glucose and Casamino acids preheated to 68 °C was

added, and the incubation temperature was adjusted to 42 °C. The culture pH was maintained between 7 and 7.4 by the periodic addition of 10 M-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 CO_2 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 Ultrasonics 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-acetic 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 concentrations were determined by e.l.i.s.a. (Prosser *et al.*, 1990). rTROPO was iodinated by using Iodo-beads at the supplier's suggested conditions; unincorporated ^{125}I was removed by gel-filtration chromatography on Bio-Gel P6 (Bio-Rad).

Bovine fibroblast culture and cell adhesion

Nuchal-ligament fibroblasts were grown from explants of fetal tissue as described by Mecham *et al.* (1981). All experiments utilized cells from passages 1–3. Fibroblast cultures were used within 2 days of visual confluence. At the time of the assay, fibroblast cultures (10 cm tissue-culture dishes) were digested with trypsin (5 ml) at 37 °C [final concentrations 0.025% (w/v) trypsin, 116 mM-NaCl, 5 mM-KCl, 1 mM-NaH_2PO_4, 6 mM-dextrose, 26 mM-$NaHCO_3$, 0.25 mM-EDTA, pH 7.4] to produce a single cell suspension; the trypsin was inhibited by the addition of turkey egg-white trypsin inhibitor (final concn. 0.2 mg/ml). Fibroblasts were washed three times with Dulbecco's modified Eagle's medium containing high glucose, low bicarbonate and 0.5% ovalbumin and resuspended at (2–4) × 10^5 cells/ml.

The adhesive properties of the proteins were studied by coating 96-well vinyl e.l.i.s.a. plates with 100 μl of protein dissolved in PBS/well for 1.5 h at 37 °C. After washing with PBS, the remaining binding sites of the wells were blocked by the addition of HDBSA for 2–2.5 h at 37 °C. The wells were sequentially washed with PBS and EBS. Then 100 μl of the cell suspension [(2–4) × 10^4 cells] was added to each well. After incubation at 37 °C for 2–2.5 h, unattached cells were removed by aspiration, and the wells were washed with EBS. The number of bound cells was quantified by the colorimetric assay of Landegren (1984) by using an e.l.i.s.a. reader at 410 nm. The results are presented as means ± S.D. of four determinations, corrected for cell adhesion to HDBSA.

Antibody preparation

Rabbit polyclonal anti-tropoelastin antibodies and monoclonal antibodies to tropoelastin were prepared as previously described (Mecham & Lange, 1982; Wrenn *et al.*, 1986). Antibodies were purified by the octanoic acid technique and resuspended at a final concentration of 5 mg/ml (McKinney & Parkinson, 1987). For Western-blot analysis, antisera were preincubated with *E. coli* to remove endogenous anti-*E. coli* antibodies (Mierendorf *et al.*, 1987).

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 pR1T2T (Nilsson *et al.*, 1985*a*,*b*). To align the reading frames of the Protein A′ gene and the cDNA, an oligonucleotide linker was joined to the 5′ end of the tropoelastin sequence. 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 pR1T2T) and extends to the *C*-terminal stop codon of the tropoelastin cDNA.

In *E. coli* N4830-1, transcription of the Protein A′ gene of pR1T2T 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 of *E. coli* proteins with non-specific immunoglobulin can be 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 pR1T2T- nor pSE76-containing *E. coli* produced a protein detected by either non-specific rabbit immunoglobulin or anti-tropoelastin antibodies (Figs. 2*a* and 2*b*). After induction, *E. coli* containing pSE76, but not pR1T2T, produced a 71 kDa protein on silver-stained gels (Fig. 2*c*), which was detected on Western blots with non-specific immunoglobulin (Fig. 2*a*). Detection by BA4, a mouse monoclonal antibody reacting with the VGVAPG sequence of tropoelastin, verified that the tropoelastin component was present (Fig. 2*b*). 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. 2*a* and 2*b*).

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. 3*a*; Nilsson *et al.*, 1985*a*; 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. 1*b*). Since there is no methionine within the

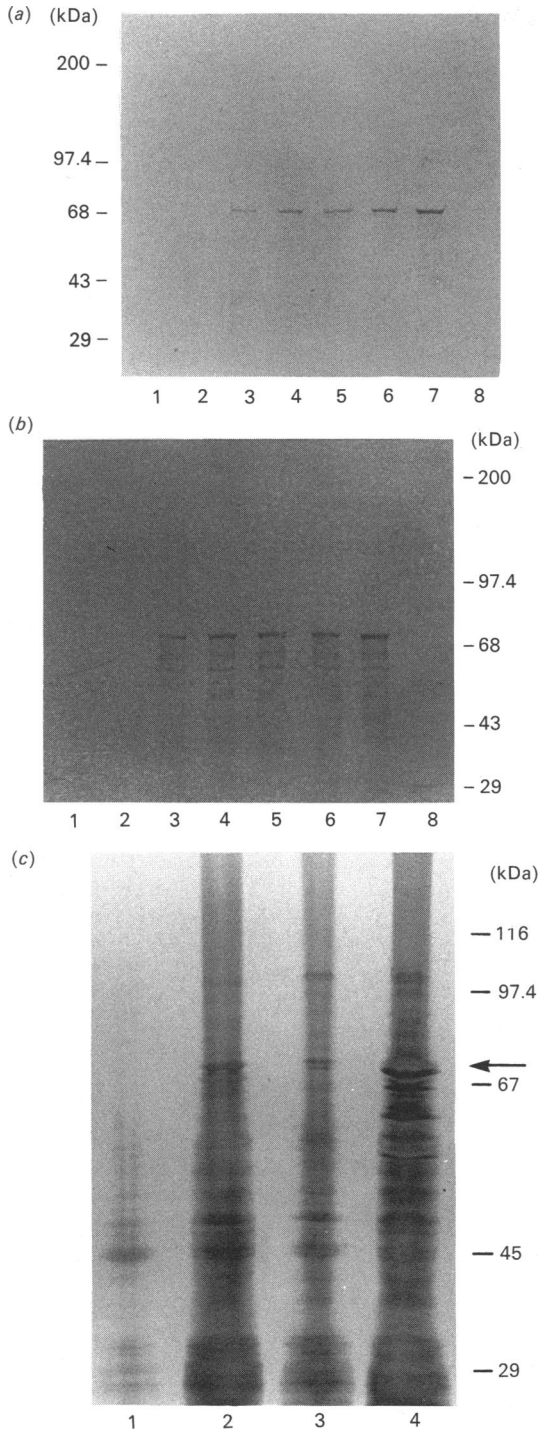

Fig. 2. Expression of Protein A–tropoelastin fusion protein by *E. coli*

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.

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. 3*a* and 3*b*). 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. 3*c*). The quantity of rTROPO isolated, as determined by e.l.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

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.

Protein concn. (μg/ml)	Cell adhesion (A_{410})	
	rTROPO	Protein A′
5	0.11±0.03	0.02±0.03
10	0.99±0.13	0.03±0.01
20	1.50±0.05	0.04±0.05

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 immunoglobumin 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.

Antibody concn. (μg/ml)	Cell adhesion (A_{410})		
	rTROPO		FCS
	BA4	Ascites	BA4
0	1.42±0.03	1.45±0.05	1.26±0.1
0.5	1.04±0.13	1.33±0.05	1.26±0.15
5	0.04±0.05	1.38±0.04	1.26±0.02
50	0.07±0.02	1.43±0.04	1.29±0.05

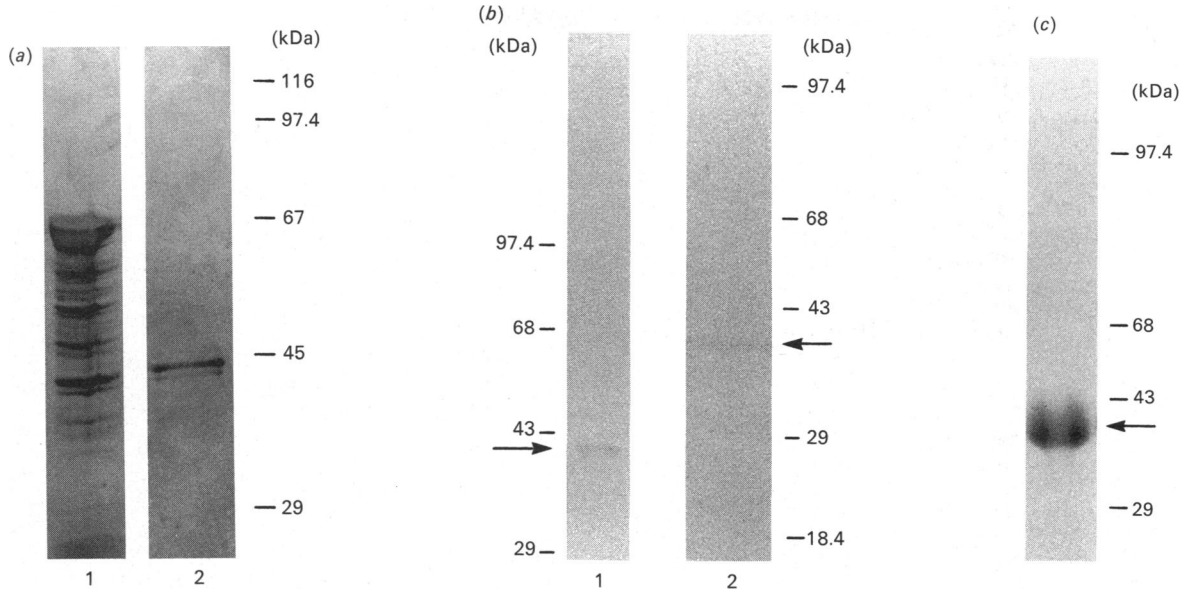

Fig. 3. Isolation and characterization of rTROPO

(*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%-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, 1984*a*). Unlike late-gestation fibroblasts, these fibroblasts do not synthesize tropoelastin or demonstrate chemotaxis to tropoelastin (Mecham *et al.*, 1981, 1984*b*; Parks *et al.*, 1988*a*,*b*). While both early-gestation [estimated gestational age 90 days (FCL_{90})] and late-gestation fibroblasts [estimated gestational age 270 days (FCL_{270})] 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 (A_{410} for $FCL_{90} = 0.74 \pm 0.16$; A_{410} for $FCL_{270} = 1.66 \pm 0.02$). 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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