The secretion of tropoelastin by chick-embryo artery cells

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Chymotryptic fingerprint analyses of tropoelastin *a* and tropoelastin *b* demonstrated a very close relationship between these two polypeptides synthesized in a cell-free system under the direction of chick-embryo polyribosomal mRNA. A similar study on tropoelastin polypeptides extracted in their hydroxylated and under-hydroxylated forms from artery cells incubated with [³H]valine in the absence and presence of $\alpha\alpha'$ -bipyridine or 3,4-dehydropyrrolidine confirmed this close relationship and suggested that tropoelastins *a* and *b* are likely to be the products of a single gene. Pulse-chase experiments in which the synthesis and secretion of tropoelastin by artery cells were monitored demonstrated that, after a pulse with [³H]proline, the polypeptides rapidly appeared in the medium and the half-time of tropoelastin secretion was approx. 30 min. Further pulse-chase studies, in which [³H]tropoelastin contents of subcellular fractions were determined, showed that rough and smooth microsomal fractions contained maximal amounts of tropoelastin at different times. The quantity of tropoelastin in the smooth-microsomal fraction was always only a small proportion of that in the rough-microsomal fraction, suggesting rapid translocation of the polypeptides to the plasma membrane. Incubation of the cells with 0.1 mM-colchicine did not markedly alter the rate of secretion or the distribution of tropoelastin between the subcellular fractions, whereas when 1 μ M-monensin was included in the incubations the polypeptides were retained in the rough microsomal fraction. The results are consistent with the proposal that tropoelastin may follow a pathway of secretion from rough endoplasmic reticulum to the plasma membrane via secretory vesicles.

The fibrous protein elastin is first synthesized as a soluble precursor known as tropoelastin, and cell-free translation studies with elastin mRNA extracted from embryonic chick tissues have demonstrated that the primary translation products are two polypeptides designated tropoelastin *a* and tropoelastin *b*, of *M_r* approx. 73000 and 70000 respectively (for review see Foster *et al.*, 1983). The suggestion that these two polypeptides might represent two genetically distinct tropoelastins (Foster *et al.*, 1980, 1981; Karr & Foster, 1981; Barrineau *et al.*, 1981) remains to be confirmed, but the possibility that they might represent the products of different mRNA molecules arising from differential post-transcriptional processing of a single gene transcript has been proposed (David-

son *et al.*, 1984). Studies in our laboratory have demonstrated that tropoelastins *a* and *b* are synthesized on membrane-bound polyribosomes and discharged into the lumen of the endoplasmic reticulum with co-translational removal of a signal peptide (Saunders & Grant, 1984). The subsequent route of secretion of the tropoelastin polypeptides remains ill-defined, for electron-microscopic and cytochemical studies have not provided a definitive view of the secretory pathway of elastin (Ross & Klebanoff, 1971; Gerritty *et al.*, 1975; Thyberg *et al.*, 1979; Damiano *et al.*, 1981).

The present paper describes investigations on the relationship between tropoelastin *a* and tropoelastin *b* synthesized by matrix-free chick-embryo artery cells and by cell-free translation of elastin mRNA from chick arteries. Further studies have probed the intracellular translocation of the newly

Abbreviation used: SDS, sodium dodecyl sulphate.

synthesized tropoelastin polypeptides and give some insight into factors influencing elastin secretion.

Experimental

Materials

Fertile eggs, obtained from Northern Biological Supplies, St. Annes, Lancashire, U.K., were incubated for 17 days at 37°C in a Westernette Egg Incubator (Western Incubators Ltd., Chelmsford, Essex, U.K.). L-[5-³H]Proline (26 Ci/mmol) was purchased from Amersham International, Amersham, Bucks., U.K., and L-[3,4-³H]valine (60 Ci/mmol) was from New England Nuclear Chemicals, Dreieich, W. Germany. Sheep anti-(chick soluble elastin) serum was generously given by Dr. B. Sykes, University of Oxford, U.K. Rabbit anti-sheep IgG was purchased from Miles Laboratories, Stoke Poges, Slough, Bucks., U.K. Triton X-405, DL-3,4-dehydroproline and imidazole were from Sigma Chemical Co., Poole, Dorset, U.K. Acrylamide, *NN'*-methylenebisacrylamide, SDS, and other chemicals (of AnalaR grade) were supplied by BDH Chemicals, Poole, Dorset, U.K.

Preparation and incubation of cells

Matrix-free cells were isolated from the major thoracic arteries of 17-day chick embryos by the procedures of Schofield *et al.* (1977). The cells were incubated for 30 min (approx. 5×10^7 cells/ml) at 37°C in modified Krebs medium (Dehm & Prockop, 1971) and then labelled with either [³H]proline (20 µCi/ml) or [³H]valine (20 µCi/ml). The labelling period was terminated by addition of unlabelled amino acid (2 mg/ml) and the incubation was terminated by cooling on ice. In pulse-chase experiments a labelling period of 4 min was used when the total [³H]tropoelastin content of cells was being measured, but in other experiments a 15 min labelling period was necessary to obtain sufficient labelling to detect tropoelastin in subcellular fractions.

Peptide 'mapping'

The primary structures of tropoelastins *a* and *b* synthesized in a nuclease-treated reticulocyte-lysate cell-free system under the direction of polyribosomal mRNA isolated from chick-embryo arteries (Saunders & Grant, 1984) were compared with tropoelastin polypeptides synthesized by matrix-free artery cells in short-term culture. The cellular tropoelastin polypeptides from cells labelled with [³H]valine for 60 min were isolated by extraction in electrophoresis sample buffer (Laemmli, 1970). Previous studies have shown that the extent of hydroxylation of proline residues in

chick tropoelastin can influence their electrophoretic migration (Saunders & Grant, 1984), and therefore, to effect an appropriate comparison with the non-hydroxylated cell-free products, unhydroxylated [³H]valine-labelled tropoelastins *a* and *b* were obtained by incubating artery cells in the presence of 1 mM- $\alpha\alpha'$ -bipyridine (Saunders & Grant, 1984) or DL-3,4-dehydroproline (100 µg/ml) (Schein *et al.*, 1977). The [³H]valine-labelled polypeptides were separated by SDS/polyacrylamide-slab-gel electrophoresis and subjected to chymotryptic digestion, with subsequent peptide 'mapping' in a second dimension by using the techniques of Cleveland *et al.* (1977). The labelled proteins were denatured under reducing conditions, separated in a 10%-polyacrylamide gel, and stained as described by Foster *et al.* (1980). The tropoelastin *a* and *b* bands were cut out of the gel by reference to the positions of the stained bands. The gel pieces were equilibrated with 0.125 M-Tris/HCl buffer, pH 6.8, containing 1% (w/v) SDS and 30% (v/v) glycerol, then placed in adjacent slots of a 15%-acrylamide gel and overlaid with 200 µl of 0.125 M-Tris/HCl buffer, pH 6.8, containing 1% SDS, 20% glycerol and chymotrypsin (100 µg/ml). Buffer containing 10% glycerol and Bromophenol Blue tracking dye was layered over the sample and electrophoresis was performed at 20 mA/gel until the dye front reached the separating gel. The power was switched off for 30 min to allow enzyme digestion to occur, and then the resulting peptides were separated by continuing the electrophoretic separation until the dye front reached the bottom of the gel. The peptides were detected by fluorography as described previously (Saunders & Grant, 1984).

Subcellular fractionation of cells

In experiments to ascertain which subcellular organelles were involved in the secretion of tropoelastin polypeptides, cells pulse-labelled with [³H]proline were homogenized and fractionated as described below. Matrix-free cells suspended at 4°C in 5 mM-imidazole buffer, pH 7.5, containing 0.25 M-sucrose were homogenized with 75 strokes at speed setting 7 of a motor-driven glass/Teflon Potter-Elvehjem homogenizer (Tri-R Instruments, New York, NY, U.S.A.) of 2 ml capacity and 0.9–1.5 mm clearance. The homogenate was centrifuged at 600 *g*_{av.} for 5 min, in 1.5 ml Eppendorf centrifuge tubes, in an MSE Minor centrifuge to sediment nuclei and cell debris. The nuclear pellet was re-homogenized with 15 strokes at speed setting 7. This homogenate was re-sedimented at 600 *g*_{av.} for 5 min and the pellet was resuspended briefly and re-sedimented to yield the nuclear fraction. The pooled supernatants were centrifuged at 10000 *g*_{av.} for 10 min in a MSE 21

centrifuge in the 8×50 rotor by using adaptors and 10 ml tubes. The pellet was suspended, and then re-centrifuged to give a pellet containing mitochondria and lysosomes. The combined post-mitochondrial supernatants were adjusted to 15 mM-CsCl, and the resulting suspension was layered over 1.3 M-sucrose in 5 mM-imidazole buffer containing 15 mM-CsCl, as described by Dallner (1963). After centrifugation at $105000g_{av}$ for 3 h in the 10×10 ml titanium angle rotor of an MSE Super-speed 75 ultracentrifuge, the rough-endoplasmic-reticulum fraction was obtained as a pellet at the bottom of the tube. The smooth-endoplasmic-reticulum fraction at the interface between the two sucrose layers was collected with a Pasteur pipette, diluted with 0.25 M-sucrose in imidazole buffer and pelleted by centrifugation at $105000g_{av}$ for 1 h.

Assay of markers for subcellular fractions

Protein assay. Protein was determined by the biuret method as described by Layne (1957) with bovine serum albumin as standard.

RNA assay. RNA was determined by the method of Munro & Fleck (1966).

Enzyme marker assays. Assays for succinate dehydrogenase and uridine diphosphatase were performed as described by Harwood *et al.* (1974). UDP-galactose:*N*-acetylglucosamine galactosyl-transferase was assayed as described by Morré (1971).

Determination of [3 H]tropoelastin

Relative concentrations of [3 H]tropoelastin were measured by electrophoresis and fluorography as described by Saunders & Grant (1984). Fluorograms were scanned with an LKB laser densitometer at 632.8 nm. Peak areas were measured with a Hewlett-Packard integrator.

Results

Characterization of intracellular tropoelastin polypeptides

Previous studies have shown that the elastin mRNA prepared from chick artery cells directs the synthesis of two polypeptides, tropoelastin *a* and tropoelastin *b*, when translated in cell-free systems (Foster *et al.*, 1980; Krawetz *et al.*, 1983). When these two closely spaced bands are excised from a polyacrylamide gel and re-electrophoresed individually, they continue to exhibit different electrophoretic mobilities (Fig. 1). Tropoelastins *a* and *b* appear to be immunologically similar, and their relationship one to another was examined by subjecting the separate products of cell-free translation to limited chymotryptic digestion followed by analysis by the procedures of Cleveland *et al.* (1977). The peptide 'maps' generated from [3 H]va-

Fig. 1. Fluorogram of the [3 H]valine-labelled polypeptides derived from tropoelastin *a* and tropoelastin *b* by partial proteolytic cleavage

Matrix-free artery cells (2.2×10^7) were incubated for 1 h in modified Krebs medium (2 ml) in the absence or in the presence of DL-3,4-dehydropioline (100 μ g/ml) with 50 μ Ci of [3 H]valine. Cells were collected by centrifugation at 1200 g for 10 min; the pellets were solubilized in electrophoresis sample buffer (250 μ l). Cell-free [3 H]tropoelastin products were synthesized by translation of total polyribosomal mRNA (25 μ g) extracted from chick arteries in the reticulocyte-lysate cell-free system (100 μ l) for 1 h at 30°C in the presence of [3 H]valine (50 μ Ci). Samples of the [3 H]polypeptides synthesized by matrix-free cells (20 μ l) or in the cell-free system (25 μ l) were separated by SDS/polyacrylamide-gel electrophoresis on 10%-acrylamide gels. Bands corresponding to tropoelastin *a* and tropoelastin *b* were excised, and the [3 H]valine-labelled proteins were subjected to proteolytic cleavage and electrophoresis on 15%-acrylamide gels, followed by fluorography. The fluorogram shows the peptide fragments derived by cleavage of (a) tropoelastin *a* and (b) tropoelastin *b* using 20 μ g of chymotrypsin: 1, products of cell-free translation; 2, non-hydroxylated cellular tropoelastins; 3, hydroxylated cellular tropoelastin. The migration positions of undigested tropoelastin (a) (TEa) and tropoelastin (b) (TEb) are indicated, together with peptides x, y, z of interest (see the text).

line-labelled tropoelastins *a* and *b* were very similar and, although some variation in the relative intensities of the individual bands was observed, the overall profiles suggest that a close relationship exists between these two polypeptides (Fig. 1).

Nevertheless, it was consistently found that cleavage of tropoelastin *a* with chymotrypsin yielded a small number of peptides which had no counterpart in the digest of tropoelastin *b*, and vice versa. For example, bands *y* and *z* in the digest of tropoelastin *b* do not correspond to equivalent bands in tropoelastin *a*, whereas band *x* from tropoelastin *a* does not correspond to a band in tropoelastin *b* (Fig. 1). These observations suggest that the differences between the two tropoelastins are probably explained by one of the polypeptides having additional amino acid sequences, although their location within the polypeptide cannot be predicted.

Peptide maps were also prepared of tropoelastins *a* and *b* isolated from intact artery cells labelled for 60 min with [³H]valine. In order to make a direct comparison with the unhydroxylated products of cell-free translation, it was necessary to minimize hydroxylation of prolyl residues by incubation of the artery cells in the presence of 1 mM- $\alpha\alpha'$ -bipyridine or 0.88 mM-3,4-dehydropyrrolidine (Schein *et al.*, 1977). The individual tropoelastin polypeptides prepared from cells incubated under these two conditions were essentially identical in their fragmentation patterns, but again it was possible to distinguish a limited number of peptides in chymotryptic digests of tropoelastin *a* which were not present in the digest of tropoelastin *b*, and vice versa (Fig. 1). It is also noteworthy that these peptide maps of unhydroxylated intracellular tropoelastins were very similar to those obtained with the tropoelastins derived by cell-free translation.

When these experiments were repeated with the hydroxylated tropoelastins isolated from artery cells, it was observed that the hydroxylated species migrated as a single broad band with a slightly slower electrophoretic mobility than the unhydroxylated tropoelastins. Consequently hydroxylated tropoelastins *a* and *b* could not be efficiently separated or extracted from the electrophoretogram. For this reason the band of hydroxylated

tropoelastins was cut in half to yield fractions of higher and lower mobilities, and these fractions were subjected to the chymotryptic 'finger-printing' procedures (Fig. 1). The peptide profiles generated show a close similarity to those produced from the unhydroxylated tropoelastins, and small differences observed in the mobilities of individual bands can possibly be explained by differences in the degree of hydroxylation of the respective peptides. Some differences in the intensity of the bands common to both species may reflect different cleavage rates achieved by chymotrypsin in these procedures and particularly in relation to differences between the hydroxylated and non-hydroxylated proteins.

Characterization of artery cell fractions

Digestion of 17-day chick-embryo thoracic arteries with bacterial collagenase and trypsin yielded a population of cells comprising predominantly immature smooth-muscle cells having a fibroblastoid morphology. Electron-microscopic examination revealed these fibroblastic cells to have extensive rough endoplasmic reticula typical of secretory cells, but the smooth endoplasmic reticulum and Golgi were not readily distinguished. In contrast, endothelial cells which were present as a minor population (<10% of cells), were observed as sheets of cells joined by tight junctions, and in these cells pronounced smooth endoplasmic reticula and Golgi were observed. Artery cells were homogenized and fractionated by differential centrifugation as described above and the fractions were assayed for RNA, protein and appropriate marker enzymes (Table 1).

The nuclear fraction was shown by microscopy to be contaminated with some whole cells and other large pieces of cellular debris. The mitochondrial fraction contained almost all the succinate dehydrogenase activity (Table 1), and by electron microscopy this fraction was shown to be rich in mitochondria and lysosomes, and also contained large subcellular particles, including a proportion

Table 1. *Characterization of subcellular fractions of artery cells*

The artery cell homogenate and subcellular fractions were assayed for RNA, protein and appropriate enzyme markers as described in the text. The distribution of each marker in the various particulate fractions is presented as a percentage relative to the concentration in the crude homogenate. The cytosolic fraction was not assayed.

Fraction	RNA (%)	Succinate dehydrogenase (%)	Uridine diphosphatase (%)	UDP-galactosyl-transferase (%)	Protein (%)
Homogenate	100	100	100	100	100
Nuclear	4	3	0.5	0.5	16
Mitochondria	13	57	13	6	13
Rough microsomal	40	2	31	30	23
Smooth microsomal	6	6	8	9	6

of large ribosome-studded vesicles. The purity of the rough microsomal fraction was confirmed by its high content of RNA and uridine diphosphatase activity (Table 1); and electron microscopy of this fraction showed it to be enriched in ribosome-studded vesicles and free ribosomes, with minimal contamination by other cellular components. In contrast, the smooth microsomal fraction consisted almost exclusively of small rounded vesicles and, although the specific activity of UDP-galactose: *N*-acetylglucosamine galactosyltransferase (a marker for Golgi in other tissues; Schachter *et al.*, 1970) in this fraction was the highest, the enzyme was recovered mainly in the rough microsomal fraction. It is not clear to what extent this result represents contamination of the rough microsomal fraction with Golgi-derived vesicles or a different location of this enzyme in connective-tissue cells.

Secretion of tropoelastin

Tropoelastin polypeptides are the major products of chick-embryo artery cells (Rosenbloom & Cywinski, 1976; Uitto *et al.*, 1976), and these components can be readily resolved from the other artery cell products by polyacrylamide-gel electrophoresis. Incubation of artery cells with [3 H]proline or [3 H]valine results in preferential labelling of the tropoelastin polypeptides because of the high proportion of these residues in tropoelastin, and quantification of the intracellular tropoelastin polypeptides is possible by analysing fluorograms of the separated 3 H-labelled polypeptides. In the following experiments tropoelastin polypeptides were quantified as a single component, for they were extracted in the hydroxylated forms and under these conditions tropoelastins *a* and *b* could not be resolved electrophoretically.

Pulse-chase experiments were conducted with matrix-free artery cells labelled with [3 H]proline, and the fate of the newly synthesized tropoelastin polypeptides was monitored. In the initial experiments a pulse label of 4 min was followed by a chase period of 18 min, and care was taken to include 10% (v/v) foetal-calf serum in the incubation medium to minimize the degradation of secreted [3 H]proline-labelled tropoelastin polypeptides. Newly synthesized [3 H]tropoelastin, identified by immunoprecipitation with anti-(soluble elastin) antiserum, was detected in the medium 4–6 min into the chase period, and after approx. 8 min the secretion of [3 H]tropoelastin was observed to follow a linear time course over the remainder of the chase period (Fig. 2).

In parallel experiments cells were pulse-labelled with [3 H]proline for 4 min, followed by an extended chase period of 125 min, during which time the intracellular content of [3 H]tropoelastin polypeptides was assessed by densitometry of the

Fig. 2. Release of [3 H]tropoelastin into the medium by matrix-free artery cells in culture

Cells (5×10^7 /ml) were preincubated for 30 min, then pulse-labelled for 4 min in modified Krebs medium with [3 H]proline (20 μ Ci/ml). The labelling period was terminated by addition of unlabelled proline and foetal-calf serum to give final concentrations of 2 mg/ml and 10% (v/v) respectively. Samples of medium (0.5 ml) were treated with anti-(soluble elastin) serum to precipitate tropoelastin as described previously (Saunders & Grant, 1984). The pellet was dissolved in 0.1% SDS (1 ml) and the radioactivity measured by liquid-scintillation counting at an efficiency of 45%. Each point represents the mean $\pm$ S.E.M. for seven experiments.

tropoelastin polypeptide bands on a fluorogram. Fig. 3 shows that the amount of intracellular [3 H]tropoelastin reaches a peak at 10 min after the start of the labelling period and then, concomitant with the linear rate of appearance of tropoelastin polypeptides in the medium (see Fig. 2), there is a linear rate of disappearance of the intracellular tropoelastin over the first 40 min of the chase period. The half-time of secretion of tropoelastin was calculated to be approx. 30 min, on the basis of plots of the percentage of [3 H]tropoelastin remaining intracellularly against time. No account was taken of [3 H]tropoelastin polypeptides which may be degraded intracellularly, but the failure to detect any increase in low- M_r 3 H-labelled components during the chase period suggested that intracellular degradation of newly synthesized tropoelastin was minimal.

Intracellular transport of tropoelastin and the influences of colchicine and of monensin

Previous studies in this laboratory (Saunders & Grant, 1984) have established that tropoelastin mRNA is associated with membrane-bound polyribosomes in embryonic-chick artery cells and that the nascent tropoelastin polypeptides undergo vectorial release into the lumen of the rough

Fig. 3. Effects of monensin and of colchicine on the intracellular contents of [^3H]tropoelastin

Matrix-free artery cells ($5 \times 10^7/\text{ml}$) were preincubated in modified Krebs medium, and after 30 min monensin ($1 \mu\text{M}$) or colchicine ($100 \mu\text{M}$) was added and incubation was continued for a further 20 min before pulse-labelling with [^3H]proline ($20 \mu\text{Ci}/\text{ml}$) for 4 min. The labelling period was terminated by addition of unlabelled proline to a concentration of $2 \text{ mg}/\text{ml}$. Samples ($200 \mu\text{l}$) of the cultures were removed at intervals up to 125 min after the beginning of the labelling period; the cells were harvested by centrifugation and then dissolved in electrophoresis sample buffer ($60 \mu\text{l}$). Samples ($30 \mu\text{l}$) was subjected to SDS/polyacrylamide-gel electrophoresis and the radioactivity in the intracellular tropoelastin bands was quantified by fluorography and laser densitometry. Labelling was carried out under the following conditions: Δ , $100 \mu\text{M}$ -colchicine; $\square$, $1 \mu\text{M}$ -monensin; $\circ$, control.

endoplasmic reticulum. In order to assess which cellular compartments are involved in the intracellular translocation of tropoelastin, artery cells pulse-labelled with [^3H]proline for 15 min and chased for up to 120 min were subjected to subcellular fractionation, and the amounts of tropoelastin present in the fractions were determined.

Initially tropoelastin was found in the rough microsomes, but, within 15–30 min of the start of the chase period, the amount of [^3H]tropoelastin polypeptides in this fraction began to fall and a concomitant increase was detected in the smooth microsomal fraction (Fig. 4). The nuclear fraction contained low amounts of tropoelastin polypeptides, whereas their content in the mitochondrial fraction was lower than, but followed that of, the rough microsomal fraction, consistent with contamination of the mitochondrial fraction with a proportion of rough microsomal fraction (results not shown).

The relationship between the tropoelastin content of the rough and smooth microsomal fractions was investigated further by using colchicine (0.1 mM) and monensin ($1 \mu\text{M}$), agents known to influence the secretion of proteins (Harwood *et al.*,

1976) and proteoglycans (Mitchell & Hardingham, 1982) at the concentrations used. Under the conditions employed here, incubation of matrix-free artery cells with either colchicine or monensin decreased the overall synthesis of tropoelastin by approx. 40%. Monensin markedly decreased the overall rate of tropoelastin secretion and resulted in an intracellular accumulation of tropoelastin polypeptides (Fig. 3). In contrast, colchicine consistently appeared to have little effect on tropoelastin secretion, and there was no obvious evidence of an intracellular accumulation of tropoelastin polypeptides (Fig. 3), although it is well documented that under identical conditions procollagen polypeptides accumulate in the smooth microsomal fraction (Olsen & Prockop, 1974; Harwood *et al.*, 1976). When these agents were used in cell-fractionation studies, it was found that colchicine decreased the amounts of [^3H]tropoelastin in the subcellular fractions, but did not significantly alter the relative distributions of tropoelastin between the fractions. However, when monensin was included in the incubation mixtures, tropoelastin polypeptides were retained in the rough microsomal fraction, and the amount in the smooth microsomal fraction was decreased (Fig. 4).

Discussion

The identity of tropoelastin as the biosynthetic precursor of insoluble elastin fibres is well documented, but the nature of tropoelastin itself and the number of elastin genes remain to be fully resolved. It has been suggested that two elastin genes may be differentially expressed, for the proportions of the two elastin-related polypeptides, tropoelastins *a* and *b*, differ significantly between lung and aortic tissues during chick embryonic development (Barrineau *et al.*, 1981). However, in the present study of elastin synthesis in chick-embryo arteries, two tropoelastin polypeptides were observed only when the unhydroxylated species were examined. When hydroxylated tropoelastins were subjected to SDS/polyacrylamide-gel electrophoresis, the microheterogeneity introduced by hydroxylation of the molecules was sufficient to mask the small difference in the mobilities of tropoelastins *a* and *b*, and it is noteworthy that attempts to enhance the separation by adding reticulocyte lysate to the system as described by Foster *et al.* (1980) were unsuccessful in our hands. 'Fingerprint' analysis of [^3H]valine-labelled tropoelastins *a* and *b* revealed a very close identity, but some differences were observed (Fig. 1), which can be best explained by the hypothesis that tropoelastin *a* contains a peptide sequence absent from tropoelastin *b*. Such a conclusion is consistent with the observation that

Fig. 4. Intracellular localization of newly synthesized tropoelastin

Matrix-free artery cells ($6.5 \times 10^7/\text{ml}$) were preincubated for 30 min in modified Krebs medium in the presence or absence of $1 \mu\text{M}$ -monensin, then pulse-labelled for 15 min with [^3H]proline ($20 \mu\text{Ci}/\text{ml}$). The labelling period was terminated by addition of unlabelled proline to a concentration of $2 \text{ mg}/\text{ml}$. Samples (1.4 ml) of the culture were removed at intervals up to 120 min after the beginning of the labelling period, and both rough and smooth microsomal fractions were obtained as described in the Experimental section. Subcellular fractions were dissolved in electrophoresis sample buffer ($200 \mu\text{l}$) and samples ($50 \mu\text{l}$) were subjected to SDS/polyacrylamide-gel electrophoresis and fluorography. The fluorogram shows (A) rough microsomal fractions and (B) smooth microsomal fractions prepared after chase periods of (1) 15 min, (2) 30 min, (3) 45 min, (4) 60 min, (5) 90 min and (6) 120 min, in (i) control incubations and (ii) in the presence of monensin ($1 \mu\text{M}$). The arrow indicates the migration position of hydroxylated tropoelastin polypeptides.

the mobilities of the higher- M_r peptides in the limited chymotryptic digest show greatest differences and that most of the low- M_r peptides are common to both polypeptides. Evidence that the proposed extra sequence is not the signal peptide which has been lost from tropoelastin *b* is indicated by studies (Karr & Foster, 1981; Krawetz *et al.*, 1983) demonstrating that tropoelastins *a* and *b* have similar *N*-terminal signal-peptide sequences. This latter observation, taken together with the 'peptide mapping' studies in Fig. 1, suggest that the two tropoelastin polypeptides are probably homologous and not the products of separate genes. Indeed, it is now evident that a single gene can produce transcripts and translation products of different lengths (Carlson & Botstein, 1982; Schwarzbauer *et al.*, 1983). However, further studies on the chick elastin gene and its expression will be necessary before it can be established that tropoelastins *a* and *b* arise by differential post-transcriptional or translational processing of a single elastin gene.

The assembly of the tropoelastin precursors on membrane-bound polyribosomes and their discharge into the lumen of the endoplasmic reticulum with co-translational removal of the signal peptide (Saunders & Grant, 1984) is consistent with the 'signal hypothesis' of Blobel (1977) and suggests that tropoelastin is likely to follow the classical route of secretion (Palade, 1975). However, in view of the failure of previous electron-microscopic, cytochemical and immunochemical studies (Ross & Klebanoff, 1971; Gerrity *et al.*, 1975; Thyberg *et al.*, 1979; Damiano *et al.*, 1981, 1984) to reach a consensus on the intracellular route of elastin secretion, the subcellular-fractionation studies described above were undertaken. Short-term pulse-chase studies with matrix-free artery cells demonstrated that the secretion of tropoelastin was quite rapid, and, in experiments where the rate of release of intracellular [^3H]tropoelastin polypeptides was assessed, the half-time of secretion of tropoelastin was determined to be approx. 30 min. This value is somewhat lower than the 60 min half-life calculated by Kao *et al.* (1982) in analogous experiments. The reason for this difference is unclear, but Kao *et al.* (1982) used much longer labelling periods, and their measurements of [^3H]tropoelastin polypeptides were based on the less specific butanol/propanol extraction procedure (Sandberg *et al.*, 1975). In further studies the [^3H]tropoelastin contents of subcellular fractions obtained from pulse-labelled artery cells were determined. In the rough microsomal fraction [^3H]tropoelastin polypeptides were present in greatest quantity at the beginning of the chase (Fig. 4), confirming our previous studies showing that newly synthesized tropoelastin is co-transla-

tionally inserted into the lumen of the rough endoplasmic reticulum (Saunders & Grant, 1984). However, significant amounts of [^3H]tropoelastin were not detected in the smooth microsomal fraction until later, when the content of the rough microsomal fraction began to fall. Thus the highest amounts of [^3H]tropoelastin in the smooth microsomal fraction were present 15–45 min into the chase period, when the tropoelastin content of the rough microsomal fraction was decreasing most rapidly. However, the amounts of tropoelastin in the smooth microsomal fraction were always less than 10% of the maximal amounts in the rough microsomal fraction (Fig. 4). In this context it is of interest that the kinetic studies of Rothman *et al.* (1980) on secretory vesicles have shown that there is a rapid turnover of these clathrin-coated vesicles, and consequently, as would be expected for any transient species, these vesicles contain at any one time only a modest amount of the protein destined for secretion. If such vesicles are the major [^3H]tropoelastin-rich component of the smooth microsomal fraction, it might therefore be expected that the amount and half-life of [^3H]tropoelastin in this fraction would be small. In other pulse-chase experiments in which a 4-min labelling period was used (results not shown), it was found that the time lag before [^3H]tropoelastin appeared in the smooth microsomal fraction was similar (10–15 min) to that required for tropoelastin release into the medium (Fig. 2). Since the smooth microsomal fraction comprises mainly smooth vesicles generated by homogenization of smooth endoplasmic reticulum, Golgi and secretory vesicles, the data again suggest a very rapid transit of tropoelastin through one or more of these organelles.

In further studies, monensin, an inhibitor of the transfer of secreted proteins from *cis* to *trans* Golgi elements (for review see Ledger & Tanzer, 1984), was added to the incubations. It was found that the trapped tropoelastin remained in association with the rough microsomal fraction, whereas amounts in the smooth microsomal fraction were decreased. These observations lead to the conclusion that most of the tropoelastin in the rough endoplasmic reticulum is not transferred via the Golgi apparatus. Since tropoelastin does not undergo any post-translational modifications associated with the Golgi apparatus, it might be expected to be able to by-pass this organelle. The different effects of colchicine and monensin on the secretion of elastin might therefore be explained by their relative influence on the budding of secretory vesicles from the rough endoplasmic reticulum. Indeed, Thyberg *et al.* (1979) have shown that electron-dense 'elastin-like' material is present in vesicles budding off the rough endoplasmic reticulum in close

proximity to the plasma membrane in cells from rat aorta. The biochemical evidence presented here suggests that tropoelastin can follow a secretory pathway from rough endoplasmic reticulum to the plasma membrane via secretory vesicles, but further work will be required to establish the relative importance of this pathway in the normal secretion of tropoelastin.

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