The Route of Secretion of Procollagen

THE INFLUENCE OF $\alpha\alpha'$ -BIPYRIDYL, COLCHICINE AND ANTIMYCIN A ON THE SECRETORY PROCESS IN EMBRYONIC-CHICK TENDON AND CARTILAGE CELLS

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1. Embryonic-chick tendon cells were pulse-labelled for 4 min with [^{14}C]proline and the ^{14}C -labelled polypeptides were chased with unlabelled proline for up to 30 min. Isolation of subcellular fractions during the chase period and their subsequent analysis for bacterial collagenase-susceptible ^{14}C -labelled peptides demonstrated the transfer of procollagen polypeptides from rough to smooth microsomal fractions and thence to the extracellular medium. Parallel analyses of Golgi-enriched fractions indicated the involvement of this organelle in the secretory pathway of procollagen. Sodium dodecylsulphate/polyacrylamide-gel electrophoresis of the ^{14}C -labelled polypeptides present in the Golgi-enriched fractions demonstrated that the procollagen polypeptides were all present as disulphide-linked pro- γ components. 2. When similar kinetic studies of the intracellular transport of procollagen were conducted with embryonic-chick cartilage cells almost identical results were obtained, but the rate of translocation of cartilage procollagen was significantly slower than that observed for tendon procollagen. 3. When hydroxylation of procollagen polypeptides was inhibited by $\alpha\alpha'$ -bipyridyl, the nascent polypeptides accumulated in the rough microsomal fraction. 4. When cells were pulse-labelled for 4 min with [^{14}C]proline and the label was chased in the presence of colchicine, secretion of procollagen was inhibited and an intracellular accumulation of procollagen ^{14}C -labelled polypeptides was observed in the Golgi-enriched fractions. 5. The energy-dependence of the intracellular transport of procollagen was demonstrated in experiments in which antimycin A was found to inhibit the transfer of procollagen polypeptides from rough to smooth endoplasmic reticulum. 6. It is concluded that procollagen follows the classical route of secretion taken by other extracellular proteins.

Studies carried out in the last decade on the site of synthesis and the secretory route taken by extracellular proteins have established a general pattern which commences with the elaboration of the polypeptide chains on ribosomes attached to the membrane of the rough endoplasmic reticulum. The segregation of secretory proteins is achieved by the vectorial release of the polypeptides into the cisternae of the rough endoplasmic reticulum, from which they are transported to the plasma membrane via the smooth endoplasmic reticulum and Golgi elements. The elucidation of this pathway has generally involved a combination of morphological analysis of intact cells and tissues and the application of biochemical techniques to monitor quantitatively the transport of the polypeptides through the subcellular compartments (for reviews see Campbell, 1971; Jamieson, 1972; Scharff, 1975; Palade, 1975).

Because of the inherent problems in homogenizing connective tissues and the consequent difficulties in obtaining homogeneous subcellular fractions, the

biochemical approach to studying procollagen secretion has been somewhat restricted (for review see Grant & Prockop, 1972*a,b,c*). Until recently, therefore, studies on the route of procollagen secretion have relied almost entirely on morphological and radioautographic approaches. On the basis of such techniques several possible routes for procollagen transport have been proposed (Revel & Hay, 1963; Ross & Benditt, 1965; Cooper & Prockop, 1968; Salpeter, 1968; Reith, 1968; Frank & Frank, 1969; Trelstad, 1971; Weinstock, 1972; Hay & Dodson, 1973; Weinstock & Leblond, 1974; Scherft & Heersche, 1975), but confirmation of those earlier studies which indicated that procollagen secretion occurs via the classical route described above has been obtained in studies using ferritin-conjugated antibodies against tendon procollagen (Olsen & Prockop, 1974; Olsen *et al.*, 1975; Nist *et al.*, 1975).

Matrix-free cells derived from embryonic-chick leg tendons and sternal cartilages have found considerable application in studies of the mechanisms

involved in procollagen biosynthesis (for reviews see Grant *et al.*, 1975; Prockop *et al.*, 1976), and the establishment of conditions for their homogenization and fractionation (Harwood *et al.*, 1974*d*, 1975*a*) has suggested the suitability of these cell systems for detailed biochemical analyses of procollagen assembly and secretion at the subcellular level. In the present study the routes of secretion of tendon and cartilage procollagens have been investigated and evidence is presented that supports the view that procollagen follows the secretory route taken by other extracellular proteins such as albumin, α_1 -acid glycoprotein, amylase and immunoglobulins.

Experimental

Materials

Fertile eggs obtained from Mytholmroyd Hatcheries, Hebden Bridge, W. Yorks., U.K., were incubated for 17 days at 37°C in a moist atmosphere in a Westernette Egg Incubator (Western Incubators Ltd., Chelmsford, Essex, U.K.). [^{14}C]Proline (290mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Chymotrypsin, trypsin, pancreatic ribonuclease A, colchicine and antimycin A were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Sephadex G-50 was supplied by Pharmacia Ltd., London W.5, U.K., and the preparation of highly purified bacterial collagenase was a generous gift from Dr. V. F. Lee-Own of this department. All other materials and reagents were from sources previously described (Harwood *et al.*, 1974*d*, 1975*a*).

Isolation and incubation of cells

Cells were isolated from the leg tendons and sterna of 17-day-old chick embryos as described previously (Dehm & Prockop, 1971, 1973; Harwood *et al.*, 1974*d*, 1975*a*). Cells were incubated at concentrations of approx. 2×10^7 cells/ml of modified Krebs medium (Dehm & Prockop, 1971) and in the experiments conducted with cartilage cells the medium was always supplemented with 10% (v/v) foetal calf serum. The cells were preincubated for 30min at 37°C then labelled for 4min with [^{14}C]proline, and in experiments where the transport through the cell of the procollagen ^{14}C -labelled polypeptides was to be studied, [^{12}C]proline was added to a concentration of 100 $\mu\text{g}/\text{ml}$ and incubation continued for up to 45min. At various intervals during this chase period portions were removed from the incubation mixture and rapidly cooled to 4°C. Further protein synthesis was inhibited by the addition of cycloheximide to 100 $\mu\text{g}/\text{ml}$. The cells were sedimented at 1200*g* for 5min and resuspended in the buffer appropriate to the subcellular fractionation procedures to be carried out.

In studies on the intracellular transport of procollagen synthesized under conditions where hydroxylation was inhibited, 0.3 mM- $\alpha\alpha'$ -bipyridyl was present throughout both the preincubation and pulse-chase periods. The fate of procollagen ^{14}C -labelled polypeptides was also examined when colchicine or antimycin A was added to the incubation medium to a final concentration of 0.1 or 0.01 mM respectively immediately after the 4min-labelling period.

Subcellular fractionation of cells

In order to study the secretory route of procollagen it was necessary to isolate subcellular fractions of rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi elements and cytosol. The homogenization procedures and fractionation schemes described by Harwood *et al.* (1974*d*, 1975*b*) were used. Because of the restricted number of cells available it was not possible within one experiment to isolate each of the above fractions for every time-point. Accordingly, tendon cells taken at 5, 10, 15 and 30min of the chase period were fractionated to obtain rough and smooth microsomal fractions and those cells chased for 10, 20 and 25min were fractionated to obtain Golgi and cytosol fractions. In a second identical experiment rough and smooth microsomal fractions were isolated from cells chased for 5, 20 and 25min and Golgi and cytosol fractions obtained from cells chased for 5, 10, 15 and 30min. Thus within the two experiments each fractionation scheme had a common time-point, i.e. 5min for the rough microsomal fraction and 10min for the Golgi fraction. The value (d.p.m. of collagenase-susceptible ^{14}C -labelled peptides/ 1×10^8 cells) obtained for the rough microsomal fraction at 5min in the second experiment was 95% of that of the initial experiment whereas that of the Golgi fraction at 10min was 93% of the corresponding sample in the first experiment. Therefore, by a minor adjustment, it was possible to relate the values obtained in the second experiment to those of the first and hence complete the series of time-points for the various subcellular fractions.

In similar studies with cartilage cells the methodology described above was followed except that the time of the chase period was extended to 45min because the time for procollagen secretion in cartilage cells is significantly longer than that for tendon cells (Dehm & Prockop, 1973).

Analysis of subcellular fractions for procollagen ^{14}C -labelled polypeptides

The passage of procollagen ^{14}C -labelled polypeptides through the subcellular compartments of tendon and cartilage cells was followed by determining the amount of radioactivity in these fractions made diffusible by the action of highly purified bacterial

collagenase. The pelleted subcellular fractions obtained as described above were resuspended in 1ml of 20mM-Tris/HCl (pH7.6) containing 0.17M-NaCl and 5mM-CaCl₂. The proteins of the cytosol fractions were precipitated by the addition of solid (NH₄)₂SO₄ to 65% saturation and then sedimented by centrifugation at 21000g for 20min. These cytosol fractions were each resuspended in 1ml of the Tris/HCl/NaCl/CaCl₂ buffer. The samples were incubated at 37°C for 24h with 20µg of bacterial collagenase purified as described by Lee-Own & Anderson (1975). This collagenase preparation had negligible proteolytic activity towards a [³H]tryptophan-labelled substrate prepared from chick embryos (Lee-Own & Anderson, 1975). Over 90% of the hydroxy[¹⁴C]proline was made diffusible under the above conditions and was recovered by dialysis at 4°C for a total of 48h against two changes of 50ml of water. The diffusate was rotary-evaporated to dryness, redissolved in 4ml of water and a portion (1ml) counted for radioactivity in 10ml of Triton X-100 toluene scintillant at an efficiency of 72% (Harwood *et al.*, 1974c).

Determination of the direction of discharge of nascent procollagen ¹⁴C-labelled polypeptides synthesized in the presence of αα'-bipyridyl

Tendon and cartilage cells were preincubated in the presence of 0.3mM-αα'-bipyridyl and labelled with [¹⁴C]proline for 4min. Cells were homogenized in a glass-Teflon motor-driven homogenizer in 0.25M-sucrose in 0.05M-Tris/HCl buffer (pH7.5) containing 0.025M-KCl and 0.5mM-MgCl₂ and microsomal fractions were isolated (Harwood *et al.*, 1974d). The susceptibility of the ¹⁴C-labelled peptides to limited proteolysis was examined by using a solution of trypsin and chymotrypsin (3mg of each dissolved in 10ml of sucrose/Tris/KCl/MgCl₂ buffer at 4°C) prepared immediately before use. Microsomal pellets resuspended in sucrose/Tris/KCl/MgCl₂ buffer (1ml) were incubated at 4°C in the absence and presence of the enzyme mixture (final concn. 50µg/ml of each enzyme). These incubations were also conducted in the presence of a mixture of 1% (w/v) sodium deoxycholate and 2% (v/v) Triton X-100. The reaction mixture was incubated for 5h then diluted to 8ml with the above buffered sucrose solution and centrifuged at 105000g for 4h. The pellets were dissolved in 8M-urea (1ml), which stops proteolysis (Harris, 1956), and 0.25mg of pancreatic ribonuclease was added and the sample incubated at 37°C for 15min, followed by centrifugation at 1000g for 10min. The supernatants were chromatographed on a column (60cm×2cm) of Sephadex G-50 equilibrated with 8M-urea/10mM-2-mercaptoethanol.

Results

Pathway of procollagen secretion in tendon and cartilage cells

Pulse-chase experiments similar to those carried out in studies on the secretion of other extracellular proteins (Jamieson & Palade, 1967; Glaumann & Ericsson, 1970; Jamieson & Ashton, 1973) were undertaken to examine the route of secretion of procollagen in tendon and cartilage cells. Tendon cells were incubated for 4min with [¹⁴C]proline, followed by a chase with unlabelled proline for up to 30min. During the chase period samples were taken and cells fractionated to yield rough and smooth microsomal fractions, Golgi elements, cytosol and extracellular medium. The data presented in Fig. 1(a) indicate that maximum labelling of procollagen with [¹⁴C]proline occurred at 10min in the rough microsomal fraction and there was a quantitative transfer of radioactive label to the smooth microsomal fraction which reached a maximum at 15min. Secretion of ¹⁴C-labelled procollagen into the medium commenced at approx. 20min and a peak of radioactivity was observed in this fraction at 25min. Only a very small proportion of the collagenase-susceptible ¹⁴C-labelled polypeptides was recovered in the cytosol fractions, and the fact that this radioactivity remained constant over the 30min-chase period provides evidence that the cytoplasm is not a compartment in the procollagen transport system.

In experiments conducted with embryonic chick cartilage cells where an extended chase period of 45min was used similar results were obtained (Fig. 1b). However, in these studies the maximum labelling of cartilage procollagen with [¹⁴C]proline occurred at 15min in the rough microsomal fraction, at 25min in the smooth microsomal fraction, and at 40min in the medium. No significant amount of label was observed in the cytosol fractions at any point in the chase period.

These results are similar to those obtained in analogous studies on the secretion of amylase (Jamieson & Palade, 1967), albumin (Glaumann & Ericsson, 1970), immunoglobulins (Scharff, 1975) and α₁-acid glycoprotein (Jamieson & Ashton, 1973), and demonstrate the transfer of newly synthesized [¹⁴C]procollagen from the rough to the smooth endoplasmic reticulum. In considering the observation that the maxima of labelling of smooth microsomal and the Golgi-enriched fractions are coincident (see Figs. 1a and 1b), it is important to appreciate that these cell types are devoted predominantly to the synthesis of extracellular protein, and are therefore likely to have extensive rough endoplasmic reticulum and well-developed Golgi apparatus [as indicated in the electron micrographs of tendon cells presented by Olsen & Prockop (1974) and Olsen *et al.* (1975)]. Consequently application of the method of Dallner

Fig. 1. Translocation of procollagen through subcellular compartments of tendon and cartilage cells

(a) Duplicate samples of approx. 18×10^8 tendon cells were incubated at 37°C with $25 \mu\text{Ci}$ of $[^{14}\text{C}]$ proline in 125 ml of modified Krebs medium. After 4 min unlabelled proline was added to a final concentration of $100 \mu\text{g/ml}$ (final incubation vol. 130 ml) and incubation was continued for a further 30 min. At intervals during the chase period samples (15 ml) were removed from the incubation medium, cooled to 4°C , made $100 \mu\text{g/ml}$ in cycloheximide and the cells collected by centrifugation. The cells were homogenized and fractionated to yield appropriate subcellular fractions which were analysed for the presence of procollagen ^{14}C -labelled polypeptides as described in the text. (b) Approx. 8×10^8 cartilage cells (in duplicate) were incubated at 37°C with $25 \mu\text{Ci}$ of $[^{14}\text{C}]$ proline in 85 ml of modified Krebs medium containing 10% (v/v) foetal calf serum. After 4 min, unlabelled proline was added to a final concentration of $100 \mu\text{g/ml}$ (final incubation vol. 90 ml) and the incubation was continued for a further 45 min. Samples (9 ml) were withdrawn at appropriate intervals and subcellular fractions isolated and assayed as above. ●, Rough microsomal fraction; ○, smooth microsomal fraction; ▲, Golgi fraction; △, cytosol; ■, medium.

(1963) to separate rough- and smooth-endoplasmic-reticulum fractions is likely to result in a large proportion of the smooth microsomal fraction from these cells comprising Golgi-derived vesicles. This problem is possibly accentuated by the relatively vigorous homogenization conditions required to fracture these matrix-free cells (Harwood *et al.*, 1974d), but by using the procedures of Schachter *et al.* (1970) it is possible to separate Golgi-derived vesicles from vesicles of smooth endoplasmic reticulum on the basis of membrane density. The data obtained by this latter approach (Figs. 1a and 1b) indicate the involvement of the Golgi apparatus in the secretion of procollagen. The decreased proportion of radioactivity in this Golgi-enriched fraction can be attributed to the limited recovery of Golgi elements by this procedure (Schachter *et al.*, 1970).

Harwood *et al.* (1973, 1975a) have demonstrated that in fractions of rough and smooth microsomal fractions isolated from these cells the major $[^{14}\text{C}]$ -proline-labelled species are procollagen polypeptides. In the smooth microsomal fractions these pro- α chains are virtually all linked by interchain disulphide bonds to form trimeric triple-helical procollagen molecules (R. Harwood, A. H. Merry, D. E. Woolley, M. E. Grant & D. S. Jackson, unpublished results) and are glycosylated to an extent almost equivalent to that of the secreted procollagens (Harwood *et al.*, 1975b). Analysis of the Golgi-derived fractions by sodium dodecyl sulphate/polyacrylamide-gel electro-

phoresis (Weber & Osborn, 1969) under both non-reducing and reducing conditions indicated that the Golgi elements of tendon and cartilage cells contain pro- α chains, all of which were found to be present in the pro- γ form (Figs. 2a and 2b).

Demonstration of the vectorial release of unhydroxylated procollagen polypeptides into the lumen of the endoplasmic reticulum

To assess whether unhydroxylated nascent procollagen polypeptides are directed into the lumen of the endoplasmic reticulum or towards the cytoplasm, the susceptibility of the nascent peptides to limited proteolysis was examined. If the nascent peptides were being directed across the endoplasmic-reticulum membrane then polypeptides within isolated microsomal fractions should be protected from proteolysis, whereas if the growing chains were directed towards the cytoplasm they would be subject to degradation by added proteinases (Blobel & Sabatini, 1970; Sabatini & Blobel, 1970; Harwood *et al.*, 1974b).

The results shown in Fig. 3 indicate that when microsomal fractions isolated from tendon cells incubated in the presence of $\alpha\alpha'$ -bipyridyl were treated with a trypsin/chymotrypsin mixture in the absence of detergent, minimal proteolysis occurred. Chromatography on Sephadex G-50 revealed a major peak at the void volume and a small included peak. By analogy with the studies of Sabatini & Blobel

Fig. 2. Electrophoretic analysis of procollagen ^{14}C -labelled polypeptides present in Golgi fractions isolated from tendon and cartilage cells

(a) Tendon cells (1570×10^6) were incubated for 2 h with $15 \mu\text{Ci}$ of ^{14}C proline in 110 ml of modified Krebs medium. The cells were collected by centrifugation and a Golgi fraction was isolated by the method of Schachter *et al.* (1970). The fraction was resuspended in 2 ml of 0.02 M-sodium phosphate buffer, pH 7.4, and a portion (1 ml) was alkylated and denatured in sodium dodecyl sulphate (1%, w/v) as described by Harwood *et al.* (1975a). A second portion (1 ml) was reduced with 2-mercaptoethanol and denatured with sodium dodecyl sulphate. The samples were analysed on 5% (w/v) polyacrylamide gels by the procedure of Weber & Osborn (1969). Electrophoresis was carried out for 4 h at 10 mA/tube and analysis of the gels for radioactivity was carried out on 1 mm slices obtained by using a Mickle Gel Slicer (Joyce, Loeb and Co. Ltd., Gateshead, U.K.). The slices were incubated at 50°C overnight in 0.5 ml of 30% (v/v) H_2O_2 and then 10 ml scintillant [5 vol. of toluene containing 6 g of 2,5-diphenyloxazole/litre and 0.12 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre and 3 vol. of 2-methoxyethanol] was added and the sample counted for radioactivity. (b) Cartilage cells (630×10^6) were incubated for 2 h with $15 \mu\text{Ci}$ of ^{14}C proline in 60 ml of modified Krebs medium containing 10% (v/v) foetal calf serum. A Golgi fraction was isolated and analysed by gel electrophoresis under reducing and non-reducing conditions as described above. ●, Unreduced sample; ○, reduced sample. The migration positions of procollagen standards are indicated.

(1970), the excluded peak corresponds to the large peptide fragments protected by the microsomal membranes and the included peak corresponds to the C-terminal segments, which are protected by the large ribosomal subunits of the membrane-bound polyribosomes. In contrast, when the detergent was present in the enzyme incubation the high-molecular-weight polypeptides were digested and only the small peptides corresponding to the fragments protected by the ribosomal structure were recovered on gel

Fig. 3. Susceptibility to limited proteolysis of nascent tendon procollagen polypeptides labelled in the presence of $\alpha\alpha'$ -bipyridyl

Tendon cells (1610×10^6) were preincubated for 30 min at 37°C in 25 ml of modified Krebs medium containing 0.3 mM- $\alpha\alpha'$ -bipyridyl. After pulse-labelling with $25 \mu\text{Ci}$ of ^{14}C proline for 4 min, cells were homogenized and microsomal fractions isolated. Portions of the microsomal fraction were subjected to limited proteolysis with a trypsin/chymotrypsin mixture at 4°C for 5 h in the presence and absence of detergent as described in the text. The samples were then centrifuged, treated with urea and ribonuclease, and chromatographed on a column (60 cm $\times$ 2 cm) of Sephadex G-50 equilibrated with 8 M-urea/10 mM-2-mercaptoethanol; fractions of 3 ml were collected. (a) Control incubation in the absence of detergent; (b) control incubation in the presence of detergent; (c) enzyme digestion in the absence of detergent; (d) enzyme digestion in the presence of detergent.

filtration. Because it was observed that detergent treatment itself did not affect the elution profile of the nascent peptides it can be concluded that disruption of the microsomal vesicles with detergents exposes the growing unhydroxylated procollagen peptides to proteolytic digestion.

When the above experiments were conducted with microsomal preparations isolated from cartilage cells that had been pulse-labelled with ^{14}C proline in the presence of $\alpha\alpha'$ -bipyridyl identical results (not shown) were obtained.

Studies on the intracellular transport of unhydroxylated procollagen in tendon and cartilage cells

It has been demonstrated that when nascent procollagen polypeptides growing in the large subunits of membrane-bound ribosomes emerge from the ribosomes they are transferred across the membrane into the lumen of the rough endoplasmic reticulum (Harwood *et al.*, 1974b), and the above

Fig. 4. Intracellular site of accumulation of unhydroxylated procollagen polypeptides

Experiments were conducted as described in Fig. 1, except that during the preincubation (see the text) and pulse-chase periods $\alpha\alpha'$ -bipyridyl was present at a concentration of 0.3 mM. (a) Tendon cells; (b) cartilage cells. ●, Rough microsomal fraction; ○, smooth microsomal fraction; ▲, Golgi fraction; △, cytosol; ■, medium.

results indicate that this transfer still occurs even when the process of hydroxylation is inhibited by $\alpha\alpha'$ -bipyridyl. However, it is well established that in the absence of hydroxylation, unhydroxylated procollagen polypeptides tend to accumulate intracellularly and their failure to be secreted may be attributable to their inability to assume a triple-helical conformation at 37°C (Prockop *et al.*, 1976).

Studies have been conducted to ascertain the subcellular compartment in which unhydroxylated procollagen polypeptides accumulate. Tendon and cartilage cells were preincubated in the presence of $\alpha\alpha'$ -bipyridyl for 30 min and after pulse-labelling with [^{14}C]proline for 4 min, subcellular fractions were isolated as described above at various times during a chase with [^{12}C]proline. The results presented in Fig. 4 demonstrate that when hydroxylation is inhibited in both tendon and cartilage cells, unhydroxylated procollagen ^{14}C -labelled polypeptides accumulate in the rough microsomal fraction and little transfer (less than 15%) to the smooth microsomal or Golgi fractions occurs. It should be noted that the maxima of labelling of the rough microsomal fractions of tendon and cartilage cells are identical with those obtained in experiments on the secretion of the respective procollagens (Fig. 1), indicating that the synthesis of the polypeptides and their transfer

into the lumen of the rough endoplasmic reticulum proceed at a normal rate within the duration of these experiments. The findings that unhydroxylated procollagen accumulates in the rough microsomal fractions are consistent with the observation of Olsen *et al.* (1975) in which ferritin-conjugated antibodies to procollagen were used to localize unhydroxylated molecules in the cisternae of the endoplasmic reticulum of tendon cells treated with $\alpha\alpha'$ -bipyridyl.

Influence of colchicine and antimycin A on the secretion of procollagen

The results presented above are consistent with procollagen following the secretory route from rough endoplasmic reticulum → smooth endoplasmic reticulum → Golgi apparatus → extracellular space. To investigate the mechanisms by which the procollagen molecules are translocated through these cellular compartments we have examined the effects of the antimicrotubular agent, colchicine, and the metabolic inhibitor, antimycin A, on the secretion process in cells from embryonic-chick leg tendons and sternal cartilages.

Cells were labelled for 4 min with [^{14}C]proline and chase experiments carried out in the presence of 0.1 mM-colchicine. Subcellular fractions were isolated as described above and the results of these pulse-chase studies are presented in Figs. 5(a) and 5(b). The data indicate that in the presence of this agent quantitative transfer of labelled procollagen from rough to smooth microsomal fractions occurs in both cell types. This transfer is slightly delayed by the action of colchicine, for in control tendon cells the maximum of labelling of the smooth microsomal fraction occurs at 15 min (Fig. 1a), whereas in the treated tendon cells the maximum is observed at 20 min (Fig. 5a). A similar delay occurs when cartilage cells are treated with colchicine (compare Figs. 1b and 5b). The absence of any significant transfer of labelled procollagen from smooth microsomal fractions to the extracellular medium (Figs. 5a and 5b) may imply a role for microtubules in the translocation of procollagen-containing vesicles to the plasma membrane. These observations are consistent with studies suggesting an increased intracellular accumulation of procollagen in cells incubated in the presence of antimicrotubular agents (Dehm & Prockop, 1972; Ehrlich & Bornstein, 1972; Diegelmann & Peterkofsky, 1972) and the localization of this accumulation in Golgi-associated vacuoles and vesicles (Ehrlich *et al.*, 1974; Olsen & Prockop, 1974). The coincidence of the maxima of labelling of smooth microsomal and Golgi-enriched fractions in these experiments (Figs. 5a and 5b) is further evidence that the smooth microsomal fraction comprises predominantly Golgi-derived vesicles.

Fig. 5. Influence of colchicine and antimycin A on the transcellular movement of procollagen

Experiments were conducted as described in Fig. 1 except that during the chase period either colchicine (0.1 mM) or antimycin A (0.01 mM) were present. (a) Tendon cells pulse-labelled with [^{14}C]proline and chased in the presence of colchicine; (b) cartilage cells pulse-labelled with [^{14}C]proline and chased in the presence of colchicine; (c) tendon cells pulse-labelled with [^{14}C]proline and chased in the presence of antimycin A; (d) cartilage cells pulse-labelled with [^{14}C]proline and chased in the presence of antimycin A. ●, Rough microsomal fraction; ○, smooth microsomal fraction; ▲, Golgi fraction; △, cytosol; ■, medium.

To investigate the energy requirements for intracellular transport, experiments were conducted in which cells were pulse-labelled as previously described and the chase experiments carried out in the presence of 0.01 mM-antimycin A (Fig. 5c and 5d). Inhibition of energy production during the chase period gave rise to an accumulation of procollagen ^{14}C -labelled polypeptides in the rough microsomal preparations and little transfer to the smooth microsomal fractions was observed. These results indicate the energy dependence of the mechanisms involved in the translocation of procollagen polypeptides from the rough endoplasmic reticulum to the smooth endoplasmic reticulum and Golgi elements in both tendon and cartilage cells.

Discussion

The subcellular mechanisms involved in the transport of procollagen from its site of synthesis on the rough endoplasmic reticulum to the extracellular space have been the subject of conflicting reports (Sheldon & Kimball, 1962; Revel & Hay, 1963; Ross & Benditt, 1965; Cooper & Prockop, 1968; Salpeter, 1968; Reith, 1968; Frank & Frank, 1969; Treistad, 1971; Hay & Dodson, 1973). In particular the role of the Golgi complex in the secretion of procollagen has until recently been ill-defined, probably because in the majority of collagen-synthesizing cells studied there is no distinctive system of con-

densed secretory granules as seen in the classical studies on exocrine cells of the pancreas (for review see Jamieson, 1972). However, in morphological and radioautographic studies on the highly polarized odontoblasts (Weinstock, 1972; Weinstock & Leblond, 1974) and in specific immunocytochemical studies with chick tendon (Olsen & Prockop, 1974; Olsen *et al.*, 1975) and corneal fibroblasts (Nist *et al.*, 1975) convincing evidence for the involvement of the Golgi apparatus in procollagen secretion has been described. Of course, such studies cannot provide precise quantitative and kinetic data, but in the work described here we have used a subcellular-fractionation approach which permits a kinetic analysis of the transfer of procollagen polypeptides through subcellular compartments and provides further data on the differences in the rates of translocation and secretion of tendon and cartilage procollagens (Dehm & Prockop, 1973; Harwood *et al.*, 1975a).

The results of the pulse-chase studies carried out on subcellular fractions isolated from tendon and cartilage cells (Figs. 1a and 1b) demonstrate a quantitative transfer of procollagen ^{14}C -labelled polypeptides from rough to smooth microsomal fractions and thence to the extracellular medium and, as discussed above, concomitant analyses of Golgi-enriched fractions indicated the involvement of this organelle in the secretory pathway. It should be noted that the appearance of [^{14}C]procollagen in the tendon and cartilage cell-incubation media after approx.

20 and 35 min respectively is consistent with previous estimates from continuous-labelling studies of the secretion times of these precursor molecules (Dehm & Prockop, 1972, 1973). The longer time required for the intracellular translocation and secretion of cartilage procollagen is not attributable to a delay in a particular subcellular compartment (compare Figs. 1a and 1b), although the extended period spent in the rough endoplasmic reticulum, in which proline hydroxylase, lysine hydroxylase and the collagen glycosyltransferases are predominantly localized (Harwood *et al.*, 1974d, 1975b), might account for the more extensive hydroxylation and glycosylation of cartilage collagen (Dehm & Prockop, 1973; Harwood *et al.*, 1975b).

In early studies concerned with the role of the hydroxylation process in the secretion of collagen it was found that incubation of connective-tissue cells under either anaerobic conditions or in the presence of $\alpha\alpha'$ -bipyridyl gave rise to an intracellular accumulation of unhydroxylated procollagen, termed procollagen. The significance of this accumulation and whether or not procollagen can be secreted in an undegraded form and follows the same secretory pathway as procollagen have been the subject of several sometimes conflicting studies (for review see Prockop *et al.*, 1976). In the experiments described in Fig. 3 the unhydroxylated procollagen ^{14}C -labelled polypeptides were shown to be directed from the large subunits of membrane-bound ribosomes into the lumen of the endoplasmic reticulum in a manner similar to that described in microsomal fractions from untreated cells (Harwood *et al.*, 1974b). Thus no evidence was obtained to support the suggestion (Diegelmann *et al.*, 1973) that inhibition of proline hydroxylase might result in the redirection of nascent procollagen polypeptides into the cytoplasm, and the absence of significant labelling of the cytosol fractions during the pulse-chase experiments described in Fig. 4 is also contrary to this suggestion. It is noteworthy that in these latter experiments $\alpha\alpha'$ -bipyridyl did not influence the times at which the rough microsomal fractions became maximally labelled, an observation that is consistent with the report that this chelating agent does not affect the release of nascent procollagen polypeptides (Lazarides & Lukens, 1971).

These pulse-chase experiments conducted in the presence of $\alpha\alpha'$ -bipyridyl provide the first kinetic data showing a failure to transport unhydroxylated procollagen polypeptides from the rough to the smooth endoplasmic reticulum and these observations correlate well with the studies using ferritin-conjugated antibodies against tendon procollagen (Olsen *et al.*, 1975), which demonstrated the localization of procollagen in the cisternae of the endoplasmic reticulum of chick tendon cells. This inhibition of procollagen translocation by $\alpha\alpha'$ -bipyridyl has been

attributed to the formation of stable enzyme-substrate complexes for proline hydroxylase, which is located almost exclusively in the rough microsomal fractions of these cell types (Harwood *et al.*, 1974d), has a high affinity for procollagen (for reviews see Grant & Prockop, 1972b; Prockop *et al.*, 1976). However, since hydroxyproline plays a key role in stabilizing the collagen triple helix at 37°C (Jimenez *et al.*, 1973; Berg & Prockop, 1973) and because several studies have suggested that the critical requirement for secretion may be the attainment of a triple-helical conformation (Uitto & Prockop, 1974a,b,c; Jimenez & Yankowski, 1975) the exact nature of this failure in intracellular transport remains to be evaluated.

As discussed above, these biochemical studies on the secretion of procollagen complement many of the previous morphological, radioautographic and immunocytochemical studies (Sheldon & Kimball, 1962; Revel & Hay, 1963; Frank & Frank, 1969; Trelstad, 1971; Weinstock, 1972; Hay & Dodson, 1973; Olsen & Prockop, 1974; Weinstock & Leblond, 1974; Olsen *et al.*, 1975; Nist *et al.*, 1975; Scherft & Heersche, 1975) and together this large body of evidence clearly establishes that the secretion of procollagen follows the classical intracellular pathway described for other secretory proteins. However, little information is available on how proteins destined for secretion are transported from one cellular compartment to another, but some insight into these mechanisms may possibly be gained by the use of drugs that inhibit the secretion process at specific locations. In our studies of the influence of colchicine on the intracellular transport of procollagen, the rate at which procollagen polypeptides were transferred from the rough to the smooth microsomal fractions was observed to decrease slightly and subsequent transfer to the extracellular medium was completely inhibited (Figs. 5a and 5b). This latter observation is in agreement with morphological studies in which colchicine was found to induce an accumulation of procollagen in Golgi-derived vesicles (Ehrlich *et al.*, 1974; Olsen & Prockop, 1974; Scherft & Heersche, 1975), and similar effects have been noted in studies on the secretion of a variety of proteins (Williams & Wolff, 1972; Stein & Stein, 1973; Le Marchand *et al.*, 1974; Redman *et al.*, 1975). These effects have generally been interpreted to imply the involvement of microtubules in exocytosis. However, in studies on the influence of colchicine on the release of plasma proteins from rat hepatocytes, Redman *et al.* (1975) were unable to ascribe the inhibitory effect of colchicine on plasma protein secretion to microtubule depolymerization, since the inhibition was prompt and reached its maximum long before the disruption of microtubules became morphologically detectable. In view of the other known effects of colchicine on membrane fractions (Wunderlich *et al.*, 1973;

Stadler & Franke, 1974; Wilson *et al.*, 1974) and the rapidity with which this agent influenced the secretion of procollagen in the experiments described here, further studies will be necessary to establish the involvement of microtubules in procollagen secretion.

The possibility that the intracellular transport of procollagen is dependent on a supply of energy, presumably as ATP, was first suggested by studies of the influence of the uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone, on procollagen synthesis in chick cranial bones (Ehrlich & Bornstein, 1972; Ehrlich *et al.*, 1974). More recent studies with cultured chick fibroblasts have confirmed a requirement for ATP in the transcellular movement and secretion of procollagen (Kruse & Bornstein, 1975), and in the experiments reported here the accumulation of procollagen ¹⁴C-labelled polypeptides in rough microsomal fractions of tendon and cartilage cells treated with antimycin A suggests that the first energy-requiring step resides at the point of transfer of procollagen from rough to smooth endoplasmic reticulum. Similar results have been obtained in studies of the influence of metabolic inhibitors on protein secretion in exocrine cells of the pancreas (Jamieson & Palade, 1968, 1971; Jamieson, 1972) and have led to the concept of an energy-dependent pre-Golgi lock (or lock gate) along the pathway of secretion (Palade, 1975). The observations that analogous mechanisms may exist in tendon and cartilage cells, which unlike the exocrine cells do not exhibit a storage phase during protein synthesis and secretion, suggests that this energy-dependent step may be a general feature of the process of protein secretion. However, the nature and mechanism of this initial energy-requiring process and the possible existence of subsequent energy-dependent steps in procollagen secretion remain to be investigated.

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