SECRETION OF PROCOLLAGEN : EVIDENCE FOR THE TRANSFER OF NASCENT POLYPEPTIDES ACROSS MICROSOMAL MEMBRANES OF TENDON CELLS.

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Summary: Microsomes isolated from tendon cells pulse-labelled for 4 min with [^{14}C]proline have been used to determine the direction of growth and release of nascent procollagen polypeptides. Puromycin was shown to induce the vectorial release of nascent peptides into the microsomal vesicles provided that the cofactors for proline hydroxylase were present in the incubation medium. Confirmation that the nascent chains were directed into the vesicular space was obtained by studies of their resistance to limited proteolysis under conditions where the vesicles were not disrupted by detergent action. These findings are compatible with the hypothesis that the first step in the secretion of procollagen polypeptides is their transfer from the ribosomes through the membranes of the endoplasmic reticulum into the cisternal space.

Introduction: In fibroblasts released from embryonic chick tendons by enzymic digestion (1) protein synthesis is devoted predominantly to the production of procollagen, the precursor of extracellular collagen (for review see ref. 2). The absence of any connective tissue matrix has enabled these tendon cells to be successfully fractionated into their subcellular compartments (3) and has thus permitted an analysis of some of the steps involved in the intracellular synthesis and secretion of procollagen (3-5). The fact that hydroxyproline synthesis occurs by hydroxylation of prolyl residues in nascent procollagen polypeptides (4,6,7) provides a specific means of locating collagen within the cell and recent studies with these cells indicate that procollagen polypeptides are synthesised on large membrane-bound polyribosomes (4,5).

Studies conducted with other secreted proteins such as serum proteins (8, 9), pancreatic amylase (10) and immunoglobulins (11) have lead to the general concept that secreted proteins are synthesised on membrane-bound ribosomes and then transferred into the cisternae of the endoplasmic reticulum (12). Whether or not procollagen is secreted by a similar process has been the

subject of several conflicting reports (for review see ref 13). However more recent reports of electron microscopic (14) and antibody-labelling studies (15) suggest that procollagen follows the same secretory route as amylase etc. and studies described here present biochemical evidence in favour of this thesis.

Materials and Methods: Matrix-free cells, isolated from 17 day-old embryonic chick tendons (1) were pulse-labelled in modified Krebs medium with [U-^{14}C]-proline (The Radiochemical Centre, Amersham). Cells were homogenised in a glass-teflon motor-driven homogeniser in 0.25M sucrose in 0.05M Tris-HCl buffer (pH 7.5) containing 0.025M KCl and 0.5mM $MgCl_2$. Microsomes were isolated by differential centrifugation and their purity has been established by chemical analysis, assays for enzyme markers and electron microscopy (3).

A study was made of the direction of release of nascent peptides detached from microsomes by puromycin treatment. The microsomal pellet was rinsed and resuspended in the above sucrose-TKM buffer. The suspension was incubated with shaking at 37° with ATP (0.7mM), GTP (0.17mM), GSH (13mM), creatinine phosphate (7mM) and creatinine kinase (1.1 units/ml). After a preincubation of 15 min to permit the 'healing' of disrupted vesicles (11), duplicate samples were further incubated at 37° for 10 min either with or without puromycin (Serva Fine Biochemicals Ltd., Heidelberg) at a final concentration of 0.2 mM. The samples were centrifuged at 105,000 g for one hour at 4°. The resultant supernatant I (Fig. 1), representing the incubation medium, was collected and the pellet rinsed and resuspended in phosphate-buffered saline (16) containing 0.5% (v/v) Triton X-100 and 5mM $MgCl_2$. The tubes were left for 15 min at 4° and then re-centrifuged at 105,000 g for one hour. This supernatant II, containing the contents of the microsomal vesicles, was collected together with the ribosomal pellet. The presence of [^{14}C]hydroxyproline in these fractions was determined by a specific radio-chemical procedure (17) after hydrolysis in 6N HCl for 24 hr at 110°.

The susceptibility of nascent peptides to limited proteolysis was examined (18,19). A solution of trypsin and chymotrypsin (3 mg of each dissolved just before use in 10 ml sucrose-TKM buffer at 4°) was prepared. Microsomal pellets resuspended in sucrose-TKM buffer (1 ml) were incubated at 4° ± the enzyme mixture (final concentration 50 ug/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. After an incubation period of 5 hr, the reaction mixture was diluted with sucrose-TKM buffer to 8 ml and centrifuged at 105,000 g for 4 hr. The pellets were dissolved in 8 M

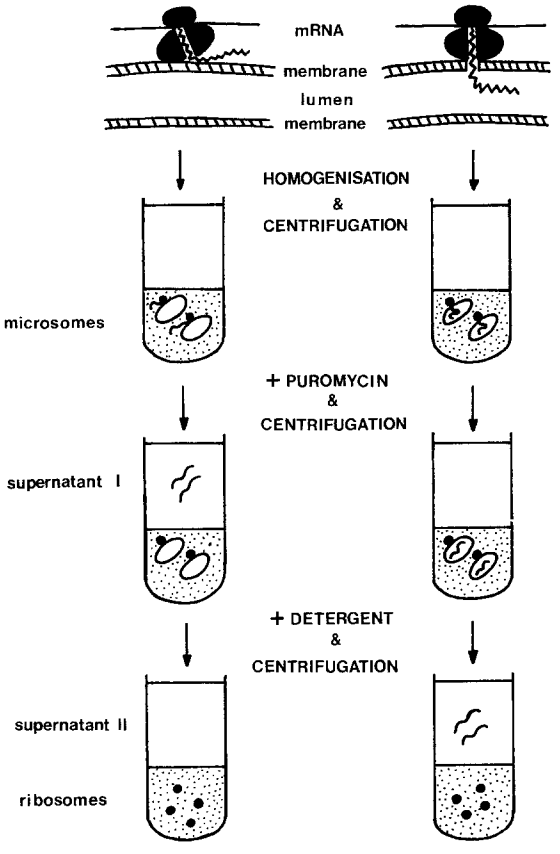

Fig. 1. Diagrammatic representation of the possible alternatives for the directional growth of nascent polypeptides attached to membrane-bound ribosomes and their release after puromycin treatment in vitro.

urea (1 ml), which stops proteolysis (20), and 0.25 mg pancreatic ribonuclease (Sigma Chemical Co., London) was added and the samples incubated at 37° for 15 min followed by centrifugation at 1000 g for 10 min. The supernatants were chromatographed on a G-50 Sephadex column equilibrated with 8 M urea and 10 mM mercaptoethanol.

Results and Discussion: The mechanisms which determine whether or not a protein is destined to be exported or retained intracellularly are not understood. Although it appears to be a general phenomenon that extracellular proteins are synthesised on membrane-bound ribosomes, the site of synthesis does not necessarily predetermine their subsequent fate (21-23) and it appears that two possible directions of discharge exist for proteins

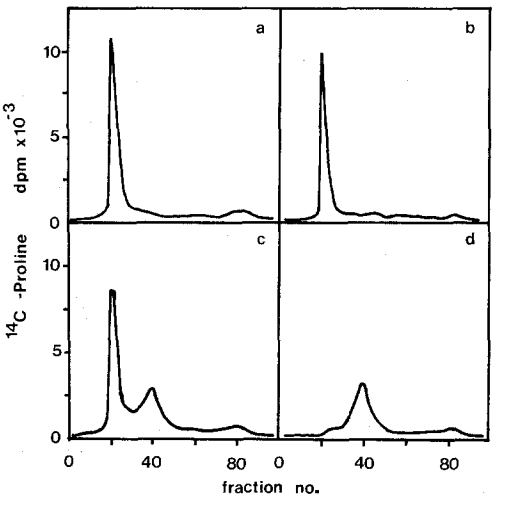

Fig. 2. Influence of detergent action on the susceptibility to limited proteolysis of nascent polypeptides present in microsomes from tendon cells labelled with [^{14}C]proline for 4 minutes. 1250 x 10^6 tendon cells were incubated with 50 μCi of [^{14}C]proline in 25 ml modified Krebs medium at 37°for 4 minutes. Microsomes were isolated and aliquots subjected to treatment with a trypsin/chymotrypsin mixture at 4° for 5 hours in the presence and absence of detergent as described in the text. The samples were then centrifuged, treated with urea and ribonuclease, and applied to a column of Sephadex G-50 (60 cm x 2.0 cm).
(a), Control without detergent; (b) Control with detergent; (c), Proteolysis without detergent; (d), Proteolysis with detergent.

synthesised on bound ribosomes (Fig. 1). The puromycin-induced release of nascent polypeptides has been used to determine the route of discharge of serum proteins (8) pancreatic amylase (10) and immunoglobulins (11). In these instances the nascent secretory protein on the membrane-bound ribosomes has, on the addition of puromycin, been shown to be released into the microsomal vesicle, which is the equivalent of the intracisternal space *in vivo*. The route of secretion of these proteins is then envisaged to be via the rough and smooth endoplasmic reticulum to the Golgi apparatus and thence to the extracellular space. Since earlier autoradiographic studies had suggested that collagen might not follow this route but be released from ribosomal complexes into the cytoplasm (see 13), we have examined the direction of release of procollagen polypeptides from their site of synthesis.

The results presented in Table 1 demonstrate that over 96% of the

[^{14}C]hydroxyproline remains with the microsomes in both control and puromycin treated samples. In initial experiments it was also found that lysis of the microsomal vesicles with detergent did not result in an increased release from the puromycin treated microsomes over that seen in the control. However, when this experiment was repeated to include the cofactors (24) for protocollagen proline hydroxylase (PPH) during the puromycin incubation, there was a significant increase in the [^{14}C]hydroxyproline released into Supernatant II. In duplicate experiments the effect of adding puromycin was to release approx. 37% and 41% of total microsomal [^{14}C]hydroxyproline into Supernatant II compared with 15-16% released in control incubations. These figures are very similar to those reported for studies on the puromycin release of other secreted proteins (8,10,11) and provides evidence that nascent procollagen peptides are directed into the lumen of the endoplasmic reticulum.

That the cofactors for PPH are required in order to observe puromycin induced release of procollagen peptides provides additional evidence that this enzyme is closely associated with the microsomal fraction (3) and is consistent with the localisation of the enzyme on the internal face of the rough endoplasmic reticulum (25). Since underhydroxylated collagen peptides form stable enzyme-substrate complexes in the absence of hydroxylation cofactors (26), puromycin may have released nascent peptides but it is probable that they could remain bound to PPH until some further hydroxylation was allowed to proceed.

Confirmation of the directional discharge of procollagen polypeptides into the intracisternal space was obtained from experiments designed to determine the susceptibility of growing peptides to limited proteolysis. If nascent peptides are being directed across the endoplasmic reticulum membrane (Fig. 1) then polypeptides within the isolated microsomes should be protected from proteolysis under the conditions described. Alternatively, if the growing chains are directed towards the cytoplasm they will be subject to degradation. The results shown in Fig. 2 indicate that when tendon cell

Table 1 : Effect of puromycin on the distribution of [^{14}C]hydroxyproline in microsomes from tendon cells pulse-labelled for 4 min with [^{14}C]proline.

Expt. No.	Incubation Conditions	Total [^{14}C]Hydroxy-proline	[^{14}C]Hydroxy-proline in Supernatant I	[^{14}C]Hydroxy-proline in Supernatant II	[^{14}C]Hydroxy-proline in Ribosomal Pellet
		dpm	%	%	%
I	Control - PPH cofactors	14770	2.3	16.3	81.4
	+ Puromycin - PPH cofactors	16321	2.4	17.5	80.1
	Control + PPH cofactors	16770	2.0	15.0	83.0
	+ Puromycin + PPH cofactors	13858	4.0	36.5	59.5
II	Control - PPH cofactors	12206	3.1	15.7	81.2
	+ Puromycin - PPH cofactors	13918	3.6	16.4	80.0
	Control + PPH cofactors	13663	3.3	15.6	81.1
	+ Puromycin + PPH cofactors	12524	5.4	41.3	53.2

In these experiments 750 x 10^6 tendon cells were incubated with 50 μCi [^{14}C]proline in 15 ml modified Krebs medium at 37° for 4 minutes. Microsomes were isolated by differential centrifugation and aliquots incubated with and without puromycin as described in the text. In certain experiments the cofactors of protocollagen prolyl hydroxylase (PPH) were present at appropriate concentrations (24) in the microsome incubation medium.

microsomes were incubated with the trypsin/chymotrypsin mixture in the absence of detergent, minimal proteolysis occurred. Chromatography on Sephadex G-50 revealed a major peak at the void volume which contained [^{14}C]hydroxyproline and a small included peak which contained no [^{14}C]hydroxyproline. By analogy with the studies of Sabatini & Blobel (16,17), the excluded peak corresponds to the large peptide fragments protected by the microsomal membranes and the included peak corresponds to the carboxy-terminal segments which are protected by the large ribosomal subunits of the membrane-bound polysomes. In contrast, when the digestion was carried out in the presence of detergents, only the small hydroxyproline-free peptides corresponding to the fragments protected by the ribosomal structure were recovered on gel filtration. Since 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 procollagen peptides to proteolytic digestion.

These results support the data from the puromycin release experiments and suggest 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.

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