Studies on the Glycosylation of Hydroxylysine Residues during Collagen Biosynthesis and the Subcellular Localization of Collagen Galactosyltransferase and Collagen Glucosyltransferase in Tendon and Cartilage Cells

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1. The glycosylation of hydroxylysine during the biosynthesis of procollagen by embryonic chick tendon and cartilage cells was examined. When free and membrane-bound ribosomes isolated from cells labelled for 4 min with [14C]lysine were assayed for hydroxy[14C]lysine and hydroxy[14C]lysine glycosides, it was found that hydroxylation took place on membrane-bound ribosomes and that some synthesis of galactosylhydroxy[14C]lysine and glucosylgalactosylhydroxy[14C]lysine had occurred on the nascent peptides. 2. Assays of subcellular fractions isolated from tendon and cartilage cells labelled for 2 h with [14C]lysine demonstrated that the glycosylation of procollagen polypeptides began in the rough endoplasmic reticulum. 14C-labelled polypeptides present in the smooth endoplasmic reticulum and Golgi fractions were glycosylated to extents almost identical with the respective secreted procollagens. 3. Assays specific for collagen galactosyltransferase and collagen glucosyltransferase are described, using as substrate chemically treated bovine anterior-lens-capsole collagen. 4. When homogenates were assayed for the collagen glycosyltransferase activities, addition of Triton X-100 (0.01 %, w/v) was found to stimulate enzyme activities by up to 45 %, suggesting that the enzymes were probably membrane-bound. 5. Assays of subcellular fractions obtained by differential centrifugation for collagen galactosyltransferase activity indicated the specific activity to be highest in the microsomal fractions. Similar results were obtained for collagen glucosyltransferase activity. 6. When submicrosomal fractions obtained by discontinuous-sucrose-density-gradient-centrifugation procedures were assayed for these enzymic activities, the collagen galactosyltransferase was found to be distributed in the approximate ratio 7:3 between rough and smooth endoplasmic reticulum of both cell types. Similar determinations of collagen glucosyltransferase indicated a distribution in the approximate ratio 3:2 between rough and smooth microsomal fractions. 7. Assays of subcellular fractions for the plasma-membrane marker 5'-nucleotidase revealed a distribution markedly different from the distributions obtained for the collagen glycosyltransferase. 8. The studies described here demonstrate that glycosylation occurs early in the intracellular processing of procollagen polypeptides rather than at the plasma membrane, as was previously suggested.

Analyses of collagens from various tissues demonstrated the existence of four genetically distinct collagen molecules, which have been designated Types I–IV (for a review, see Miller, 1973). Chemical analyses of these collagens demonstrated the presence of galactose and glucose in covalent linkage with hydroxylysine, either as galactosyl-O-β-hydroxylysine or 2-O-α-D-glucosylgalactosylhydroxylysine (for a review, see Spiro, 1972). The amount of glycosylated hydroxylysine found in different tissue collagens varies from approx. one glycosylated hydroxylysine per 1000 amino acid residues in tendon and scleral collagen to over 30 glycosylated hydroxylysine residues per 1000 amino acid residues in basement-membrane collagens (Spiro, 1969; Kefalides, 1973; Grant, 1975). The roles of these carbohydrate moieties have not been specifically defined, but the glycosylated hydroxylysines may be involved in fibrillogenesis (Morgan et al., 1970), and they have also been implicated in the processes of collagen-mediated platelet aggregation (Barber & Jamieson, 1971a,b; Bosmann, 1971; Chesney et al., 1972; Katzman et al., 1973; Kang et al., 1974).

The synthesis of these hydroxylysine glycosides has been shown to involve two enzymes, collagen UDP-galactosyltransferase and collagen UDP-glucosyltransferase, both of which require Mn2+ (Bosmann & Eylar, 1968a,b; M. J. Spiro & R. G. Spiro, 1971; R. G. Spiro & M. J. Spiro, 1971a). Evidence was presented indicating the localization of these trans-
ferases in the plasma membranes of various cell lines (Hagopian et al., 1968; Bosmann, 1969), but these analyses were carried out with a simple assay procedure in which the UDP-[14C]glucosylgalactosylhydroxylysine was incorporated into phosphotungstic acid-precipitable material with sugar-depleted collagen as substrate. This methodology has been criticized on several grounds (M. J. Spiro & R. G. Spiro, 1971; R. G. Spiro & M. J. Spiro, 1971a), but when the subcellular distribution of these enzymes was investigated by using the specific assay procedures of R. G. Spiro & M. J. Spiro (1971b), the data confirmed that these transferases were membrane-bound but their precise location could not be assessed.

We have demonstrated that in freshly isolated tendon and cartilage cells the first post-translational events in collagen biosynthesis, i.e. hydroxylation of appropriate proline and lysine residues, commence while the growing procollagen polypeptides are still attached to ribosomes (Harwood et al., 1973, 1974a,c, 1975a), and the two enzymes proprocollagen hydroxylase and protocollagen lysine hydroxylase are located predominantly in the rough endoplasmic reticulum of these cells (Harwood et al., 1974c).

To determine the subcellular site of glycosylation of hydroxylsine residues during the biosynthesis of Type I and Type II procollagens, we have examined the extent of glycosylation of procollagen polypeptides isolated from subcellular fractions of these cell types labelled under various conditions. In addition, the specific assays for collagen galactosyltransferase (M. J. Spiro & R. G. Spiro, 1971) and collagen glucosyltransferase (R. G. Spiro & M. J. Spiro, 1971a) activities have been modified to decrease the time involved in assaying the labelled glycosidic products, and these procedures have been used in the subcellular localization of the two transferases. Preliminary reports of parts of this work have already been presented elsewhere (Harwood et al., 1975b,c).

Experimental

Materials

Substrates for the assays of collagen glycosyltransferases were derived from lens capsules dissected from bovine eyes obtained from a local abattoir within 1–2h of slaughter. Pepsin (3x crystallized) and 5'-ATP were purchased from Sigma (London) Chemical Co., London S.W.6., U.K. Ion-exchange resin AG 50W-X8 (200–400 mesh) obtained from Bio-Rad Laboratories, St. Albans, Herts., U.K., was cleaned and prepared in the H+ form by the method of Moore & Stein (1951) just before use. UDP-[U-14C]-galactose (300mCi/mmol) and UDP-[U-14C]glucose (260mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Standards of glucosylgalactosylhydroxylysine[14C]lysine and galactosylhydroxylysine[14C]lysine were prepared by ion-exchange chromatography (Askenasi & Kefalides, 1972) of an alkaline hydrolysate of procollagen secreted by embryonic chick tendon cells labelled for 2h with [14C]lysine as described below. All other materials and reagents were from sources described previously (Harwood et al., 1974b,c, 1975a).

Isolation and incubation of matrix-free cells

Cells were isolated from the leg tendons and sterna of 17-day chick embryos by digestion with trypsin and partially purified collagenase under conditions similar to those described by Dehm & Prockop (1971, 1972, 1973). In the short-term pulse-labelling experiments cells were incubated at 37°C at a concentration of 5×10^6 cells/ml of modified Krebs medium (Dehm & Prockop, 1971), but when cells were incubated for 2h the concentration was 10^7 cells/ml of medium. The incubations were conducted in silicone-treated glass Erlenmeyer flasks, and cells were preincubated at 37°C for 15min before addition of the appropriate 14C-labelled amino acid. Incubations were terminated by rapid cooling to 4°C and the addition of cycloheximide to a final concentration of 100μg/ml.

Subcellular fractionation of cells

Preparation of free and membrane-bound cytoplasmic ribosomes. Cells labelled for 4min with [14C]lysine were collected by centrifugation at 1200g for 5min. The cells were resuspended in 0.25M-sucrose in 0.05M-Tris–HCl buffer, pH 7.5, containing 25mM-KCl and 0.5mM-MgCl2, and homogenized under conditions described previously (Harwood et al., 1974c). Nuclei and mitochondria were sedimented at 4°C by centrifugation at 10000g (r, 6.5cm) for 10min in the 10×10ml titanium angle rotor of an MSE Superspeed 65 ultracentrifuge. The pellet was re-homogenized, then centrifuged at 10000g for 10min, and the two supernatants were combined. Free and membrane-bound ribosomes were obtained by centrifugation on a discontinuous sucrose gradient as in the method of Blobel & Potter (1967).

Preparation of subcellular fractions by differential centrifugation. Subcellular fractions were obtained by procedures based on the method of Schachter et al. (1970). Cells were homogenized in the sucrose–Tris–KCl–MgCl2 buffer as described above and the homogenate was centrifuged at 600g for 5min in an MSE Mistral 6L centrifuge to sediment nuclei and cell debris. The nuclear pellet was resuspended in the 0.25M-sucrose buffered solution and re-homogenized. This homogenate was resedimented at 600g for 5min to yield the nuclear fraction (N) and a supernatant which was pooled with the first postnuclear supernatant. The pooled supernatants were centrifuged at 10000g (r, 6.5cm) for 10min in the 10×10ml titanium angle rotor of an MSE Superspeed 65 centrifuge to yield a pellet and a supernatant (S3). The pellet was resuspended in 0.34M-sucrose in 0.05M-
Tris–HCl buffer, pH 7.5, containing 25 mm-KCl and 0.5 mm-MgCl₂ and recentrifuged at 10000 g for 10 min to yield a mitochondrial fraction (ML) and a Golgi-enriched supernatant. This supernatant was adjusted to 0.25 mm-sucrose and centrifuged at 10000 g for 10 min to pellet the Golgi-enriched fraction (G). Supernatant (S3) was centrifuged at 105000 g (rₑₑₑ, 6.5 cm) for 1 h to obtain a microsomal pellet (M) and the cytosol fraction (C).

**Preparation of submicrosomal fractions by discontinuous gradient centrifugation.** Rough endoplasmic reticulum was separated from smooth endoplasmic reticulum by using a discontinuous-sucrose-density-gradient technique based on the method of Dallner (1963). Details of these centrifugation procedures and the characterization of the fractions obtained have been described previously (Harwood et al., 1974c).

**Analysis of subcellular fractions.** Fractions were assayed for the plasma-membrane marker, 5'-nucleotidase, by measuring the release of P₁ from 5'-AMP (Heppel & Hilmo, 1951). This assay was conducted under optimum conditions, and conditions under which product formation was proportional to time of incubation and to the quantity of enzyme protein placed in the incubation mixture.

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

**Assay for hydroxy[¹⁴C]lysine glycosides by ion-exchange chromatography.**

Cells were incubated with [¹⁴C]lysine for either 4 min or 2 h and the extents of glycosylation of hydroxy[¹⁴C]lysine in secreted procollagen, intracellular procollagen and procollagen polypeptides present in various subcellular fractions were determined. Samples were dialysed against running tap water for 18 h and alkaline hydrolysis and subsequent desalting were carried out as described by Grant et al. (1972). [¹⁴C]lysine, hydroxy[¹⁴C]lysine and hydroxy[¹⁴C]lysine glycosides were separated by a modification of the method of Askenasi & Kefalides (1972). The hydrolysates were dissolved in 0.1 m-HCl and chromatographed at pH 5.3 on the long column of a JEOI JLC 6AH amino acid analyser. Fractions (2.5 ml) were collected and samples (1 ml) were counted for radioactivity in 10 ml of scintillant as described previously (Harwood et al., 1974b).

**Determination of collagen glycosyltransferases.**

**Preparation of substrates.** Anterior-lens-capsule collagen was prepared by a procedure based on the method of Kefalides & Denduchis (1969). Anterior lens capsules dissected from adult bovine eyes were freed of cellular material by sonication in water for a total of ten 30 s periods at 4°C at auto setting 6 on an MSE Sonicator. Capsules were freeze-dried and subsequently digested with crystalline pepsin (enzyme/substrate ratio of 1:10) in 250 volumes of 0.1 m-acetic acid at 20°C for 18 h. The enzymic digest was centrifuged at 40000 g for 1 h and the supernatant was dialysed for 18 h against 0.01 m-acetic acid (8 litres) at 4°C. To the sample was added solid KCl to a final concentration of 15% (w/v) and solid Na₂HPO₄ to a concentration of 0.02 m. The precipitated anterior-lens-capsule collagen was collected by centrifugation at 40000 g for 1 h, redissolved in 0.01 m-acetic acid and dialysed against 0.01 m-acetic acid as above. The collagen preparation was then freeze-dried.

The substrate for collagen galactosyltransferase was prepared by removing the glucosylgalactose disaccharide units on the anterior-lens-capsule collagen by a single Smith degradation involving periodate oxidation, NaBH₄ reduction and mild acid hydrolysis (Spiro, 1967). Approx. 100 mg of freeze-dried collagen was incubated in 20 ml of 0.02 m-sodium metaperiodate in 0.05 m-sodium acetate buffer, pH 4.5, at 4°C in the dark for 27 h. Then 0.1 m-ethylene glycol (4 ml) was added and the mixture left for 30 min at 4°C before exhaustive dialysis against water. The non-diffusible material was suspended in 15 ml of 0.1 m-sodium borate buffer, pH 8.0, to which was added 2.5 ml of 1.0 m-NaBH₄ and the reduction was allowed to proceed for 24 h at 4°C. The reaction mixture was adjusted to pH 5.0 with acetic acid, exhaustively dialysed against water, freeze-dried and then subjected to mild acid hydrolysis in 0.1 m-HCl at 80°C for 2 h. The sample was neutralized with 2 m-NaOH and used directly in the galactosyltransferase assay.

The substrate for collagen glucosyltransferase was prepared by the selective removal of glucose from the basement-membrane collagen by hydrolysis in 0.05 m-H₂SO₄ for 20 h at 100°C (R. G. Spiro & M. J. Spiro, 1971a). After hydrolysis the sample was neutralized with 2 m-NaOH and used directly in the assay.

**Collagen galactosyltransferase assay.** The incubation was carried out in polypropylene tubes (5 ml) for 2 h at 37°C in a total volume of 200 μl, which contained 15 μmol of Tris–HCl buffer, pH 6.8, 2 μmol of MnCl₂, 0.4 μmol of 2-mercaptoethanol, 10 μl of 0.1% (v/v) Triton X-100, 0.63 μCi of UDP-[¹⁴C]galactose and 3 mg of disaccharide-free lens-capsule collagen. The reaction was stopped by rapidly cooling to 4°C followed by the addition of 15 vol. of acetone. The protein precipitate was collected by centrifugation and the pellet washed a further three times each with 3 ml of cold acetone. Alkaline hydrolysis was carried out by adding 2 ml of 2 m-NaOH to the pellet in the polypropylene tubes, which were then sealed within large glass tubes and heated at 105°C for 18 h.

After hydrolysis the samples were diluted with water (10 ml) and titrated at 4°C to pH 3.5–4.0 with 1 m-HCl. The samples were applied to small columns containing 6 g of Bio-Rad AG 50W–X8 ion-exchange
resin (H⁺ form). The columns were washed with water (50ml) and the hydroxylysine[14C]glycosides were eluted with 1.5M-NH₃ (25ml). The samples were dried by rotary evaporation of the NH₃ at 40°C and final traces were removed by freeze-drying after redissolving the sample in water (4.0ml). The samples were dissolved in 1 ml of 0.1M-pyridine-acetate buffer, pH 5.0, and 100µl portions were applied to 4cm × 25cm strips of Whatman no. 1 paper and electrophoresis was carried out in 0.1M-pyridine-acetate buffer, pH 5.0, at 250V for 4.5h. Standards of galactose, UDP-[14C]galactose, galactosylhydroxy-[14C]lysine, hydroxylysine and lysine were electrophoresed on an adjacent strip. After drying at 100°C, the strips were cut into 1 cm sections and their radioactivity was determined in 10ml of toluene scintillation fluid [5g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 litre of toluene].

Collagen glucosyltransferase assay. The incubation was carried out for 2h at 37°C in a total volume of 200µl which contained 15µmol of Tris–HCl buffer, pH 6.8, 5µmol of MnCl₂, 0.4µmol of 2-mercaptoethanol, 10µl of 0.1% Triton X-100, 0.67µCi of UDP-[14C]glucose and 3mg of glucose-free lens-capsule collagen. Precipitation of the protein with acetone and subsequent alkaline hydrolysis, desalting, and electrophoresis of the samples were carried out as described for the collagen galactosyltransferase assay.

Characterization of the products formed by the glycosyltransferases. Previous assays for these enzymes (Bosmann & Eylar, 1968a,b) are subject to the criticism that the products of the enzymatic reactions were not fully characterized. The need to measure specifically the hydroxylysine[14C]glycosides was recognized by R. G. Spiro & M. J. Spiro (1971a), who introduced a paper-chromatographic separation which required 5–6 days to run. Such a time-consuming assay presents difficulties if it is to be used in a multi-step purification procedure, and with this long-term aim in view it was considered desirable to decrease the assay time without loss of specificity.

By using defined substrates of high molecular weight it was possible to precipitate the 14C-labelled products of the enzymic reactions with acetone and remove the exogenous UDP-[14C]glycoside. Of the radioactivity incorporated, not all was present as hydroxylysine[14C]glycoside, since a proportion (30%) of the 14C was not retained by the cationic resin. The major portion of the radioactive eluted by NH₃ electrophoresed in a position corresponding to standards of galactosylhydroxylysine and glucosylgalactosylhydroxylysine, although a small peak of radioactivity having a mobility intermediate between UDP-[14C]galactose and galactosylhydroxylysine was usually observed (Figs. 1a and 1b).

It should be noted that the electrophoretic procedure does not separate the [14C]glucosylgalactosylhydroxylysine from the [14C]galactosylhydroxylysine, but the identities of these labelled species synthesized in the respective enzyme assays with UDP-[14C]-glucose or UDP-[14C]galactose were confirmed by ion-exchange chromatography (Fig. 2).

Results

Analysis of procollagen polypeptides in subcellular fractions

In an attempt to establish the site of glycosylation of the hydroxylysine residues of procollagen, subcellular fractions were isolated from cells labelled

![Fig. 1. Paper electrophoresis of the products of the enzymic relations](image_url)

After alkaline hydrolysis, neutralization and desalting of the acetone-precipitable products of the enzyme reactions, a portion was applied to Whatman no. 1 chromatography paper (3cm × 25cm) and electrophoresis was carried out in 0.1M-pyridine-acetate buffer, pH 5.0, at 250V for 4.5h. After drying at 100°C the strips were cut into 1 cm sections and the radioactivity was determined as described in the text. (a) Products of galactosyltransferase; (b) products of glucosyltransferase. The positions of UDP-[14C]galactose, galactosylhydroxy[14C]lysine and hydroxylysine are shown on guide strips.
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Fig. 2. Analysis by ion-exchange chromatography of the products of the enzyme reactions

Alkaline hydrolysates of the products of the enzyme incubations were neutralized and desalted then applied to the long column of a JEOL JLC 6AH amino acid analyzer. Fractions (2.5 ml) were collected and samples (1 ml) were counted for radioactivity in 10 ml of scintillant as described previously (Harwood et al., 1974b). (a) Products of galactosyltransferase assay; (b) products of glucosyltransferase assay; (c) Standards of glucosylgalactosylhydroxy[14C]lysine, galactosylhydroxy[14C]lysine, hydroxy[14C]lysine and [14C]lysine prepared by alkaline hydrolysis of the procollagen present in the medium of embryonic-chick tendon cells incubated with [14C]lysine for 2 h at 37°C under conditions described in the text.

with [14C]lysine under steady-state conditions and analyzed for the presence of hydroxy[14C]lysine and hydroxy[14C]lysine glycosides. Initial analyses of the proteins secreted into the medium by tendon and cartilage cells incubated for 2 h in modified Krebs medium indicated that approx. 20 and 70% of the hydroxy[14C]lysine residues were substituted in the respective procollagens (Tables 1 and 2). Concomitant analyses of the total cellular proteins revealed that approx. 15 and 60% of the hydroxy[14C]lysine residues in intracellular tendon and cartilage procollagen polypeptides were glycosylated. These values indicate that the substitution of hydroxylysine has reached over 70% of the final values observed for the secreted molecules and suggest that in both cell types the intracellular pool of unglycosylated polypeptides must be relatively small and that the glycosylation process probably commences soon after hydroxylation of appropriate lysine residues. Such a conclusion would argue for a microsomal rather than a plasma-membrane location (Hagopian et al., 1968; Bosmann, 1969) for the collagen glycosyltransferases, and this suggestion is supported by analyses of subcellular fractions (Tables 1 and 2).

When Golgi-enriched and microsomal fractions prepared by the method of Schachter et al. (1970) were assayed for hydroxy[14C]lysine and hydroxy- [14C]lysine glycosides, the results indicated that the synthesis of galactosylhydroxy[14C]lysine and glucosylgalactosylhydroxy[14C]lysine had commenced in the microsomal fractions of both tendon and cartilage cells. The proportions of hydroxylysine residues substituted with hexose in these microsomal fractions were approx. 50 and 80% of the values found in the procollagens secreted by tendon and cartilage cells respectively. In contrast, the procollagen polypeptides present in the Golgi-enriched fractions isolated from these cell types were glycosylated to an extent almost identical with the secreted molecules.

Submicrosomal fractions of tendon and cartilage cells were isolated by the discontinuous-sucrose-density-gradient procedures of Dallner (1963). Analysis of these fractions revealed that procollagen polypeptides present in the rough-endoplasmic-reticulum fractions contained galactosylhydroxy-[14C]lysine and glucosylgalactosylhydroxy[14C]lysine (Tables 1 and 2). However, the ratios of disaccharide to monosaccharide were significantly lower than the ratios determined for the extracellular procollagens, suggesting that the galactose and glucose residues are not added simultaneously but in a stepwise manner, which may be dictated by the spatial separation of the glycosyltransferases. The procollagen polypeptides in the smooth-endoplasmic-reticulum fractions were more extensively glycosylated, the proportions of free and substituted hydroxylysine and also the distribution between mono- and disaccharide units being comparable with values obtained for the Golgi-enriched fractions isolated by differential centrifugation. This latter observation probably reflects the fact that both cell types are likely to have an extensive rough endoplasmic reticulum and well developed Golgi apparatus, and therefore segregation of microsomes into rough and smooth membranes might be expected to yield smooth-endoplasmic-reticulum fractions containing a large proportion of Golgi-derived vesicles.

Demonstration of the glycosylation of nascent procollagen polypeptides on membrane-bound ribosomes

The demonstration that the synthesis of hydroxylysine in cartilage cells occurs on nascent procollagen
Table 1. Extent of glycosylation of hydroxylysine in procollagen polypeptides present in subcellular fractions from tendon cells

In Expt. I, 10⁸ tendon cells were incubated with 10μCi of [¹⁴C]lysine for 2h at 37°C. Medium and cells were separated, dialysed against running tap water and freeze-dried. In Expts. II and III, 11×10⁸ and 9×10⁸ cells respectively were incubated with 50μCi of [¹⁴C]lysine and subcellular fractions obtained as described in the text. All the samples were hydrolysed in 2m-NaOH at 105°C for 18h before analysis on the amino acid analyser.

<table>
<thead>
<tr>
<th>Expt. Fraction</th>
<th>Total [¹⁴C] radioactivity (d.p.m.)</th>
<th>Unsubstituted [¹⁴C]Hyl (d.p.m.)</th>
<th>Gal-[¹⁴C]Hyl (d.p.m.)</th>
<th>Glc-Gal-[¹⁴C]Hyl (d.p.m.)</th>
<th>Free Hyl (%)</th>
<th>Glycosylated Hyl (%)</th>
<th>Distribution of glycosylated units (%)</th>
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<tr>
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<td>255200</td>
<td>54160</td>
<td>3360</td>
<td>11120</td>
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<td>18.9</td>
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<td>173200</td>
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<td>19510</td>
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<td>17.4</td>
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Table 2. Extent of glycosylation of hydroxylysine in procollagen polypeptides present in subcellular fractions from cartilage cells

In Expt. I, 1.1×10⁸ cartilage cells were incubated with 10μCi of [¹⁴C]lysine for 2h at 37°C. Medium and cells were separated, dialysed against running tap water and freeze-dried. In Expts. II and III, 7×10⁸ and 6×10⁸ cells respectively were incubated with 50μCi of [¹⁴C]lysine for 2h and subcellular fractions obtained as described in the text. All the samples were hydrolysed in 2m-NaOH at 105°C for 18h before analysis on the amino acid analyser.

<table>
<thead>
<tr>
<th>Expt. Fraction</th>
<th>Total [¹⁴C] radioactivity (d.p.m.)</th>
<th>Unsubstituted [¹⁴C]Hyl (d.p.m.)</th>
<th>Gal-[¹⁴C]Hyl (d.p.m.)</th>
<th>Glc-Gal-[¹⁴C]Hyl (d.p.m.)</th>
<th>Free Hyl (%)</th>
<th>Glycosylated Hyl (%)</th>
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<td>8557</td>
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Table 3. Analysis of hydroxylation of lysine and glycosylation of hydroxylysine in nascent procollagen polypeptides

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</table>

polypeptides attached to membrane-bound ribosomes (Harwood et al., 1975a) and the above findings that the synthesis of hydroxylysine glycosides commences in the rough endoplasmic reticulum of both tendon and cartilage cells prompted an investigation of the extent of glycosylation, if any, of the hydroxylysine residues of nascent polypeptides. Tendon and cartilage cells were labelled for 4 min with [14C]lysine, and free and membrane-bound ribosomes were isolated by the method of Blobel & Potter (1967). That the 14C-labelled procollagen peptides isolated after this labelling period represent nascent chains was demonstrated in previous studies (Harwood et al., 1974a, 1975a) in which the procollagen peptides were found to sediment with large polyribosomes on sucrose-density-gradient centrifugation. Analyses of free and membrane-bound ribosomes (Table 3) confirmed that the synthesis of tendon and cartilage procollagen occurs only on membrane-bound ribosomes, and that the synthesis of hydroxylysine occurs on nascent tendon procollagen polypeptides also.

The detection of galactosylhydroxy[14C]lysine and glucosylgalactosylhydroxy[14C]lysine in the membrane-bound ribosomal fractions provides the first demonstration that the glycosylation of procollagen commences while the growing chains are still attached to the ribosomes (Table 3). In the fraction from the tendon cells approx. 7% of the hydroxy[14C]lysine is substituted, of which less than 10% is present as the disaccharide unit, whereas in the cartilage sample approx. 14% of the hydroxy[14C]lysine is substituted, of which 25% is present as glucosylgalactosylhydroxy[14C]lysine.

Effect of Triton X-100 and protein concentration on the activities of the collagens glycosyltransferases

To investigate further the glycosylation of hydroxylysine residues, studies on the collagen galactosyltransferase and collagen glucosyltransferase were undertaken. Initial experiments using homogenates of the matrix-free cells indicated that the presence of 0.01% Triton X-100 in the incubation mixture stimulated the activity of both glycosyltransferases by approx. 45%. The detergent was therefore added as a routine to all subsequent assays, and (as shown in Fig. 3) the activities of these enzymes derived from both tendon and cartilage cells were proportional to the enzyme concentrations.

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Table 4. Distribution of collagen galactosyltransferase activity in subcellular fractions from tendon and cartilage cells

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>Protein content (mg)</th>
<th>[14C]Gal-Hy activity (d.p.m.)</th>
<th>Relative specific activity</th>
<th>Sp. activity (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tendon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.43</td>
<td>4.21</td>
<td>0.3</td>
<td>2.94</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.35</td>
<td>1.03</td>
<td>0.3</td>
<td>2.34</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.39</td>
<td>2.74</td>
<td>0.7</td>
<td>3.96</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>0.40</td>
<td>2.60</td>
<td>0.7</td>
<td>3.49</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>0.21</td>
<td>1.82</td>
<td>0.9</td>
<td>2.06</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>0.16</td>
<td>1.95</td>
<td>1.2</td>
<td>1.62</td>
</tr>
<tr>
<td>Cartilage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.32</td>
<td>5.18</td>
<td>1.3</td>
<td>3.88</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.37</td>
<td>1.31</td>
<td>0.7</td>
<td>2.00</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.35</td>
<td>2.34</td>
<td>0.7</td>
<td>3.14</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>0.32</td>
<td>1.98</td>
<td>0.7</td>
<td>3.04</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
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<td>1.51</td>
<td>0.8</td>
<td>1.88</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>0.16</td>
<td>1.42</td>
<td>0.8</td>
<td>1.10</td>
</tr>
</tbody>
</table>

**Distribution of collagen galactosyltransferase and collagen glucosyltransferase in subcellular fractions of tendon and cartilage cells**

Subcellular fractions obtained by the centrifugation schemes described above were assayed for protein content and collagen galactosyltransferase activity. The enzyme activity was measured in a total volume of 0.1 ml. The amount of [14C]galactose per subcellular fraction was determined as described in the Experimental section. The specific activity is the ratio of the specific activity of the subcellular fraction to that of the homogenate. The relative specific activity is the ratio of the specific activity of the subcellular fraction to that of the homogenate.

Table 5 reports the results obtained for the distribution of collagen glucosyltransferase in subcellular fractions from both tendon and cartilage cells. The results show that the two transferases are similar in their distribution patterns, but in this case slightly more of the glucosyltransferase activity (approx. 40%) is associated with the smooth-endoplasmic-reticulum fractions.

**Comparison of the distributions of the collagen glycosyltransferases and the plasma-membrane marker, 5'-nucleotidase**

Since previous studies (Hagopian et al., 1968; Bosmann, 1969) have raised the possibility that collagen glycosyltransferases may be bound to the plasma membrane, it was considered important to establish the fate of the plasma-membrane fragments in the centrifugation procedures used in the present study. Subcellular fractions obtained by differential centrifugation and by sucrose-density-gradient centrifugation were assayed for the enzyme 5'-nucleotidase which is generally considered to be a plasma-membrane marker and which has been shown to be present in plasma membranes isolated from embryonic chick tendon cells (Lehtinen et al., 1975). In both centrifugation procedures the majority of the 5'-nucleotidase activity was found in the nuclear and mitochondrial fractions (Fig. 4 and Table 6). Less than 15% of the total activity is present in the microsomal fraction, whereas this latter fraction...
Table 5. Distribution of collagen glucosyltransferase activity in subcellular fractions from tendon and cartilage cell homogenates

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>Protein Content (mg)</th>
<th>Protein Content of Glucosyltransferase (10^6 c.p.m.)</th>
<th>Relative Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tendon Homogenate</td>
<td>0.46</td>
<td>6.03</td>
<td>13.1</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.32</td>
<td>1.34</td>
<td>1.0</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.37</td>
<td>1.34</td>
<td>1.0</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>0.32</td>
<td>1.34</td>
<td>1.0</td>
</tr>
<tr>
<td>Microsomal Fraction</td>
<td>0.25</td>
<td>0.73</td>
<td>3.6</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.18</td>
<td>0.56</td>
<td>3.0</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>0.18</td>
<td>0.56</td>
<td>3.0</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>0.18</td>
<td>0.56</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Fig. 4. Distribution of collagen glucosyltransferase and 5'-nucleotidase in subcellular fractions obtained by differential centrifugation of tendon and cartilage cell homogenates

The subcellular fractions are: N, nuclei; ML, mitochondria and lysosomes; M, microsomal fraction; C, cytosol. Each of the fractions was assayed for protein content, and the collagen glucosyltransferase activity associated with the fractions is presented as a hatched histogram superimposed on the distribution of the 5'-nucleotidase activity present in these fractions. (a) Tendon cell fractions; (b) cartilage cell fractions.

contains over 55% of the collagen glucosyltransferase activity in both tendon and cartilage cells (Figs. 4a and 4b). The small amounts of 5'-nucleotidase activity present in the microsomal fractions are found to be associated predominantly with the smooth-endoplasmic-reticulum fractions (Table 6), whereas the major portion of the glycosyltransferase activities is located in the rough-endoplasmic-reticulum fractions (Tables 4 and 5).
Table 6. Distribution of 5'-nucleotidase activity in submicrosomal fractions of tendon and cartilage cells

Tendon and cartilage cells were homogenized in 0.25 M sucrose and fractionated by the method of Dallner (1963). Fractions were assayed for protein and 5'-nucleotidase activity and values are given relative to those obtained with the crude homogenate.

<table>
<thead>
<tr>
<th>Tendon</th>
<th>5'-Nucleotidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei</td>
<td>31.3</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>23.6</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>5.1</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>5.6</td>
</tr>
<tr>
<td>Cytosol</td>
<td>33.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cartilage</th>
<th>5'-Nucleotidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei</td>
<td>33.8</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>25.1</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>5.2</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>4.9</td>
</tr>
<tr>
<td>Cytosol</td>
<td>30.7</td>
</tr>
</tbody>
</table>

Discussion

During the last decade the processes involved in the synthesis and secretion of glycoproteins were studied in considerable detail and it is now generally accepted that secretory proteins are synthesized on membrane-bound ribosomes and that carbohydrate is added as they are transported from the rough endoplasmic reticulum through the smooth endoplasmic reticulum to the Golgi apparatus and out of the cell. Recent studies with matrix-free tendon and cartilage cells (Harwood et al., 1973, 1974a,d, 1975a; Olsen et al., 1973; Olsen & Prockop, 1974) have demonstrated that the synthesis and secretion of procollagen follows the general pathway of secreted glycoproteins, but the site of glycosylation of the hydroxylysine residues has not been satisfactorily defined.

In glycoprotein biosynthesis the glycosylation process frequently commences either with the addition of xylose to peptidylserine or with the addition of N-acetylglucosamine to peptidyl-asparagine, and further glycosylation occurs by the sequential addition of appropriate sugar residues catalysed by membrane-bound glycosyltransferases located along the secretory pathway. The glycosylation of collagen, however, requires that the product of translation of procollagen mRNA undergoes prior hydroxylation of peptidyl-lysine residues by protocollagen lysine hydroxylase. This latter process is found to commence while nascent procollagen polypeptides are still attached to the ribosomes (Table 3), and the molecule thus becomes a substrate for the collagen galactosyltransferase at the ribosomal level. It was originally proposed that the subsequent addition of galactose and then glucose under the direction of the specific collagen glycosyltransferases occurred just before secretion, since these enzymes were initially reported to be associated with the plasma membrane (Hagopian et al., 1968; Bosmann, 1969). However, the demonstration that glycosylation of hydroxylysine during the biosynthesis of basement-membrane collagen occurred at an early stage in the intracellular processing of this procollagen (Grant et al., 1972) did not appear to be consistent with this hypothesis.

The ability to obtain well-characterized subcellular fractions of tendon and cartilage cells (Harwood et al., 1973, 1974c, 1975a) has enabled us to re-examine the intracellular site of collagen glycosylation. Initial analyses of subcellular fractions obtained by differential centrifugation (Tables 1 and 2) indicated that procollagen polypeptides in the microsomal fraction were substantially glycosylated, suggesting that the addition of galactose and glucose commenced early in the intracellular transport of both tendon and cartilage procollagens. When submicrosomal fractions isolated by discontinuous-sucrose-density-gradient centrifugation were analysed, it was evident that polypeptides present in the rough-endoplasmic- reticulum fractions were partially glycosylated, whereas the extents of glycosylation of the polypeptides in the smooth-endoplasmic-reticulum fractions were almost the same as those of the secreted procollagens (Tables 1 and 2). These results were obtained with cells labelled under steady-state conditions and in further studies using short-term pulse-labelling conditions it was demonstrated that the addition of both galactose and glucose was initiated on growing peptide chains attached to membrane-bound ribosomes (Table 3). Thus the glycosylation of procollagen appears to occur soon after the synthesis of hydroxylsine, and it will be noted that the hydroxylysine of nascent cartilage procollagen polypeptides is substituted to a greater extent than in the tendon polypeptides, an observation which may reflect the higher activities of the glycosyltransferases in the cartilage cells (Tables 4 and 5).

The above results are at variance with the suggestion that the collagen glycosyltransferases are located on the plasma membrane (Hagopian et al., 1968; Bosmann, 1969). However, this latter suggestion was based on studies using assay systems which have since been criticized for their lack of specificity.
GLYCOSYLATION OF COLLAGEN

(R. G. Spiro & M. J. Spiro, 1971a; M. J. Spiro & R. G. Spiro, 1971) and, in the case of the galactosyltransferase, it has been pointed out (M. J. Spiro & R. G. Spiro, 1971) that the preparation of the substrate by using two successive periodate oxidations would destroy any hydroxylsine-acceptor sites in the protein. When the subcellular distribution of these enzymes was examined in rat renal-cortical tissue by using assays developed to measure specifically the synthesis of the hydroxylsine glycosides (R. G. Spiro & M. J. Spiro, 1971b), the enzymes were shown to be present primarily in a rapidly sedimenting particulate fraction. This fraction was not characterized further, although the observation that the enzyme activities were increased by the presence of Triton X-100 was in contrast with previous reports (Hagopian et al., 1968; Bosmann, 1969) and would be consistent with a vesicular location for these enzymes.

To determine the subcellular location of the collagen glycosyltransferases in the tendon and cartilage cells, the specific assay procedures (R. G. Spiro & M. J. Spiro, 1971a; M. J. Spiro & R. G. Spiro, 1971) were modified to overcome the step involving the time-consuming separation of the hydroxylsine glycosides by paper chromatography. By introducing the paper-electrophoretic procedure described above, it has been possible to decrease the time involved by a factor of 3 without any loss of specificity. It should be noted that during the preparation of this manuscript a similar modification was reported by Risteli & Kivirikko (1974).

Preliminary evidence that both the transferases are membrane-bound in tendon and cartilage cells was obtained by studies of cell homogenate assayed for these enzymes in the presence and absence of 0.01% Triton X-100. Both enzyme activities were found to be stimulated (approx. 45%) in the presence of the detergent, confirming the previous observations of R. G. Spiro & M. J. Spiro (1971b) and Risteli & Kivirikko (1974). Assays of the subcellular fractions of the two transferases indicated that less than 15% of the total activities were present in the cytosol fractions and the relative specific activities of both galactosyltransferase and glycosyltransferase were found to be highest in the microsomal fractions isolated from tendon and cartilage cells (Tables 4 and 5). When submicrosomal fractions obtained by discontinuous-sucrose-density-gradient-centrifugation procedures were assayed for these enzymic activities, the collagen galactosyltransferase was found to be distributed in the approx. ratio 7:3 between rough and smooth endoplasmic reticulum of both cell types. Similar determinations of collagen glucosyltransferase indicated a distribution in the approximate ratio 3:2 between rough and smooth microsomal fractions.

These studies appeared to eliminate the plasma membrane as the site of the transferases, but the possibility of plasma-membrane fractions contaminating the microsomal fraction could not be eliminated entirely. However, preliminary investigations of the influence of Triton-X100 on the glycosyltransferases in cell homogenates had indicated that these enzymes exhibited structure-linked latency in that they were further activated by the detergent (see above). The plasma-membrane marker 5'-nucleotidase, has been shown not to exhibit such latency in homogenates of rat liver (De Duve, 1971) and rat embryo fibroblasts (Tulkens et al., 1974), and in studies of the activity of this enzyme in tendon- and cartilage-cell homogenates we were unable to detect any stimulation of 5'-nucleotidase by the addition of Triton X-100. These results provided the first indication that the glycosyltransferases and the 5'-nucleotide had different locations and this observation was confirmed by the demonstration of a lack of coincidence between the distributions of 5'-nucleotidase and the collagen glycosyltransferases in the subcellular fractions of both tendon and cartilage cells (Fig. 4 and Table 6).

The discovery that the collagen glycosyltransferases are associated predominantly with the rough-microsomal fraction is in contrast with the more usual observations of glycosyltransferases being present in smooth endoplasmic reticulum and Golgi fractions (Fleischer et al., 1969; Schachter et al., 1970). However, several demonstrations that the initiation of the assembly of the carbohydrate side chains of glycoproteins may be a ribosomal event have previously been reported (Molnar & Sy, 1967; Sherr & Uhr, 1970; Schenkin & Uhr, 1970; Uhr & Schenkin, 1970). In our earlier studies of the synthesis of hydroxyproline and hydroxylysine in tendon and cartilage cells (Harwood et al., 1973, 1974a,c, 1975a) it was observed that these post-translational events commenced on nascent polypeptides and it now appears that the subsequent addition of galactose and glucose to the hydroxylysine residues can also occur at the ribosomal level. These observations suggest that the four enzymes responsible for the secondary modifications after the translation of procollagen mRNA may reside in a contiguous relationship as a multi-enzyme system bound to the internal face of the cisternae of the rough endoplasmic reticulum.

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References
